Human calicivirus SRSV-Ba/98/CH

Taxonomy ID: 122390
Rank: no rank
Genetic code: Translation table 1 (Standard)

Lineage(full)
Viruses; ssRNA positive-strand viruses, no DNA stage; Caliciviridae; Norovirus; Norwalk virus; Small round structured virus

Entrez records	
Database name	Direct links
Nucleotide	1
Protein	1
Taxonomy	1

ICTV homepage

Comments and questions to info@ncbi.nlm.nih.gov
Credits: Mikhail Domrachev, Scott Federhen, Carol Hotton, Detlef Leipe, Vladimir Soussov, Richard Sternberg, Sean Turner.

1: A37491. hypothetical heli...[gi:476783] BLink, Domains, Links

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LOCUS       A37491                 1737 aa            linear   VRL 17-MAR-2000
DEFINITION  hypothetical helicase/polymerase polyprotein - Southampton virus.
ACCESSION   A37491
VERSION     A37491  GI:476783
DBSOURCE    pir: locus A37491;

            summary: #length 1737 #molecular-weight 193342 #checksum 6745
            ;
            superfamily: rabbit calicivirus RNA-directed RNA polymerase
            ;
            PIR dates: 03-Mar-1994 #sequence_revision 25-Apr-1997 #text_change
            17-Mar-2000
            .
KEYWORDS    .
SOURCE      Southampton virus
  ORGANISM  Southampton virus
            Viruses; ssRNA positive-strand viruses, no DNA stage;
            Caliciviridae; Norovirus.
REFERENCE   1  (residues 1 to 1737)
  AUTHORS   Lambden,P.R., Caul,E.O., Ashley,C.R. and Clarke,I.N.
  TITLE     Sequence and genome organization of a human small round-structured
            (Norwalk-like) virus
  JOURNAL   Science 259 (5094), 516-519 (1993)
  MEDLINE   93142023
   PUBMED   8380940
  REMARK    small round-structured virus, SRSV, Norwalk virus, Norwalk-like
            virus, serotype 3
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                     /db_xref="taxon:11984"
     Protein         1..1737
                     /product="hypothetical helicase/polymerase polyprotein"
                     /note="orf1 protein"
ORIGIN
        1 malgligrtt peptgtagpp pkqqrdrppr tqeevqygmg wsdrpidqnv ksweeldttv
       61 keeildnhke wfdagglgpc tmpptyervk ddsppgeqvk wsardgvnig verlttvsgp
      121 ewnlcplppi dlrnmepase ptigdmiefy eghiyhysiy igqgktvgvh spqaafsvar
      181 vtiqpiaaww rvcyipqpkh rlsydqlkel enepwpyaai tnncfefccq vmnledtwlq
      241 rrlvtsgrfh hptqswsqqt pefqqdskle lvrdailaav nglvsqpfkn flgklkplnv
      301 lnilsncdwt fmgvvemvil llelfgvfwn ppdvsnfias llpdfhlqgp edlardlvpv
      361 ilggiglaig ftrdkvtkvm ksavdglraa tqlgqyglei fsllkkyffg gdqtertlkg
      421 ieaavidmev lsstsvtqlv rdkqaakaym nildneeeka rklsaknadp hvisstnali
      481 srismarsal akaqaemtsr mrpvvimmcg ppgigktkaa ehlakrlane irpggkvglv
      541 preavdhwdg yhgeevmlwd dygmtkildd cnklqaiads apltlncdri enkgmqfvsd
      601 aivittnapg papvdfvnlg pvcrrvdflv ycsapeveqi rrvspgdtsa lkdcfkldfs
      661 hlkmelapqg gfdnqgntpf gkgtmkptti nrlliqaval tmerqdefql qgkmydfddd
      721 rvsafttmar dnglgilsma glgkklrgvt tmeglknalk gykisactik wqakvysles
      781 dgnsvnikee rniltqqqqs vctasvaltr lraaravaya sciqsaitsi lqiagsalvv
      841 nravkrmfgt rtatlslegp prehkcrvhm akaagkgpig hddvvekygl ceteedeeva
      901 hteipsatme gknkgknkkg rgrrnnynaf srrglndeey eeykkireek ggnysiqeyl
      961 edrqryeeel aevqaggdgg igetemeirh rvfykskrk hhqeerrqlg lvtgsdirkr
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1021 kpidwtppks awadderevd ynekisfeap ptlwsrvtkf gsgwgfwvsp tvfittthvi
1081 ptsakeffge pltsiaihra geftlfrfsk kirpdltgmi leegcpegtv csvlikrdsg
1141 ellplavrmg aiasmriqgr lvhgqsgmll tganakgmdl gtipgdcgap yvykrandwv
1201 vcgvhaaatk sgntvvcavq asegettleg gdkghyaghe iikhgcgpal stktkfwkss
1261 peplppgvye paylggrdpr vtvgpslqqv lrdqlkpfae prgrmpepgl leaavetvts
1321 sleqvmdtpv pwsysdacqs ldkttssgfp yhrrknddwn gttfvrelge qaahannmye
1381 qaksmkpmyt galkdelvkp ekvyqkvkkr llwgadlgtv vraarafgpf cdaikshtik
1441 lpikvgmnsi edgpliyaeh skykyhfdad ytawdstqnr qimtesfsim crltaspela
1501 svvaqdllap semdvgdyvi rvkeglpsgf pctsqvnsin hwlitlcals evtglspdvi
1561 qsmsyfsfyg ddeivstdie fdpakltqvl reyglrptrp dksegpiivr ksvdglvflr
1621 rtisrdaagf qgrldrasie rqiywtrgpn hsdpfetlvp hqqrkvqlis llgeaslhge
1681 kfyrkisskv iqeiktggle myvpgwqamf rwmrfhdlgl wtgdrnllpe fvnddgv
//
```

Our data indicate that the ΔNL mutation increases Aβ production by augmenting cleavage at or very near the site of mutation on the amino side of Aβ. Thus this mutation likely increases all Aβ in a way that does not alter the specific site (or sites) of COOH-terminal cleavage. Although the βAPP_{717} mutations do not appear to increase overall Aβ production, these mutations on the COOH-side of Aβ may shift cleavage to favor generation of the longer Aβ's (42 to 43 residues long) that are specifically associated with senile plaque amyloid. As these longer Aβ's have biophysical properties that favor amyloid deposition (*25*), shifting the site of cleavage could result in amyloid deposition without increasing the overall amount of Aβ. Such a shift would not be detectable with our current gel system, and the longer forms might more readily form insoluble aggregates that would impair detection. Whatever the precise mechanism proves to be, it is likely, given the positive results obtained with the ΔNL mutation, that continued analysis of the βAPP_{717} mutations will ultimately reveal altered processing that is highly informative with regard to the mechanism through which these mutations produce AD.

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23. Each of our cell lines is polyclonal and was independently transfected using the episomal replicon CEP4β. Although polyclonal lines show less variation than monoclonal lines, it is nonetheless critically important to assess the level of expression (synthesis) of WT and mutant βAPPs before concluding that the processing of a mutant βAPP is altered in a way that increases Aβ release. Synthesis is best measured by quantitating the full-length βAPP that accumulates during a labeling interval sufficiently short that no appreciable degradation takes place. We labeled for 20 min, the shortest interval that permitted us to assess multiple lines in parallel in a way that was practical. Although some degradation may occur during this short interval, this method of assessing synthesis is nonetheless a reliable way to ensure that excess production of Aβ in lines expressing mutant βAPPs is due to altered processing and not to increased expression.
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26. To assemble CEP4β-695ΔNL, we used a βAPP_{695} template and oligonucleotides 1 and 2 to amplify by the polymerase chain reaction (PCR) a cDNA extending from base 1762 to 2119 followed by an adaptor sequence containing an Xba I restriction site [numbering is according to Kang *et al.* (*7*)]. Oligonucleotide sequences are shown below. The forward primer employed in this reaction (oligonucleotide 1) altered the WT nucleotides at positions 1785 and 1786 from G to T and A to C, respectively, changing the encoded amino acids immediately amino to $A\beta_1$ from lysine to asparagine and methionine to leucine, respectively. The Bgl II–Xba I fragment of this amplified cDNA was then used to replace the corresponding fragment of a βAPP_{695} cDNA (nucleotides −30 to 2119) in BSKS⁻ (Stratagene). To assemble CEP4β-695ΔI, we used a βAPP_{695} template and oligonucleotides 3 and 2 to PCR amplify a cDNA extending from 1906 to 2119 followed by an adaptor sequence containing an Xba I restriction site. The forward primer used in this reaction (oligonucleotide 3) altered the WT nucleotide at position 1924 from G to A, changing the encoded valine to isoleucine. This cDNA was gel-purified and used as a template along with a βAPP_{695} template and oligonucleotides 4 and 5 to produce a ΔI cDNA extending from base 1761 to 2119 followed by an adaptor sequence containing an Xba I restriction site. The Eco RI–Xba I fragment of this amplified cDNA was then used to replace the corresponding fragment of Bluescript βAPP_{695}. Both the full-length 695ΔNL and 695ΔI sequences in Bluescript were then subcloned into Hind III–Not I sites of CEP4β. The oligonucleotides used were (1) ACGGAGGAGATCTCTGAAGTGAATCTGGATGCAGAATTCC (2) GAGGGCCATGCCGGCCTCTAGAGTCCAACTTCAGAGGCTGCT (3) GTCATAGCGACAGTGATCATCATCAC (4) GACGGAGGAGATCTCTGAAGT, and (5) GAGGGCCATGCCGGCCTCTAGA.

27. Supported by NIH grant AG06656 and an ADRDA Zenith award. We thank A. LeBlanc for anti-C_{21}.

25 November 1992; accepted 10 December 1992

Sequence and Genome Organization of a Human Small Round-Structured (Norwalk-Like) Virus

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Small round-structured viruses (SRSVs), also known as Norwalk or Norwalk-like viruses, are the major worldwide cause of acute, epidemic nonbacterial gastroenteritis in humans. These viruses, which contain a single-stranded RNA genome, have remained refractory to molecular characterization because of the small amounts of virus in clinical samples and the absence of an animal model and an in vitro culture system. The complete genomic nucleotide sequence of an SRSV, Southampton virus, was determined. The 7696-nucleotide RNA genome encodes three open reading frames whose sequence and organization strongly support proposals that SRSVs are members of the Caliciviridae.

The term "epidemic winter vomiting disease" was first used by Zahorsky in 1929 (*1*) to describe a syndrome in humans now globally recognized as acute nonbacterial gastroenteritis (*2*). The prototypical pathogen, Norwalk virus, was identified by American workers after an outbreak of acute nonbacterial gastroenteritis in an elementary school in Norwalk, Ohio (*3*). Morphologically indistinguishable viruses have since been described in clinically similar outbreaks and have been termed Hawaii (*4, 5*), Snow Mountain (*6, 7*), and Taunton agents (*8, 9*) after the geographical locations in which they were first found. Our electron microscopy (EM) studies, performed with human convalescent sera (*10*), of virus isolates collected from laboratory-confirmed outbreaks have established at least four serotypes in the United Kingdom and the United States. We designate the Norwalk and Hawaii viruses as serotypes 1 and 2, respectively, because of their historical precedence; the prototype strains for serotypes 3 and 4 are the Snow Mountain and Taunton viruses. Others also recognize at least four serotypes (*11*). Since February 1991, serotype 3 has been the most preva-

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lent serotype, causing outbreaks of acute nonbacterial gastroenteritis in England and Wales (12).

The inability to propagate this group of viruses in vitro has severely hampered the development of diagnostic reagents. As a result, EM remains the most widely used technique for identification and diagnosis. The amorphous nature of the Norwalk-like viruses permits their differentiation from other small round viruses such as astroviruses, parvoviruses, and the classical caliciviruses and has resulted in an interim scheme for their classification (13). This scheme has entailed the introduction of the term "small round-structured virus" (SRSV) within the United Kingdom and elsewhere to differentiate this group of viruses from other small round fecal viruses. Comparative EM studies have suggested that this virus has some morphological similarities to caliciviruses (8). The caliciviruses are 35- to 40-nm particles with a characteristic cupped morphology, possessing a single-stranded RNA genome and a major structural protein of 60 to 71 kD (14). Like the caliciviruses, Norwalk virus has a single-stranded RNA genome (15, 16) and a single major capsid protein with a molecular weight of 59,000 (17).

The objective of our work here was to determine the complete nucleotide sequence of a recent SRSV isolate. A stool sample that contained characteristic SRSV particles (Fig. 1) was collected from a 2-year-old child with acute diarrhea during a recent family outbreak of acute gastroenteritis in Southampton, United Kingdom (18). The virus, hereafter designated Southampton virus, was shown to be serotype 3 by immuno-EM, and this sample was the sole source of viral RNA for cDNA synthesis and DNA sequence determination (19, 20).

The genome of Southampton virus consists of 7696 nucleotides, which does not include a 3' polyadenylate [poly(A)] tail. The GenBank accession number for the cDNA sequence is L07418. The 5' end of the RNA contains an untranslated leader sequence of

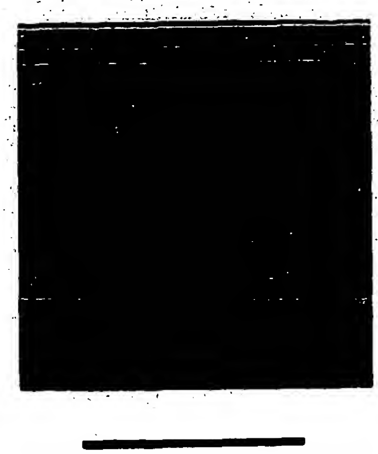

Fig. 1. Electron micrograph of SRSV serotype 3 particles. The virus was negatively stained for 2 min in 0.5% phosphotungstic acid (pH 6.0) and visualized with a Hitachi H7000 transmission electron microscope. The scale bar represents 100 nm.

ORF1

```
1     MALGLIGRTTPEPTGTAGPPPKQGRDRPPRTQEEVQYGHGWSDRPIDQHVKSWEELDTTVKEEILDNHKEWFDAGGLGPCTMPPTYERVKDDSPPGEQVK
101   WSARDGVNIGVERLTTVSGPEWNLCPLPPIDLRNMEPASEPTIGDMIEFYEGHIYHYSIYIGQGKTVGVHSPQAAFSVARVTIQPIAAWWRVCYIPQPKH
201   RLSYDQLKELENEPWPYAAITNNCFEFCCQVMNLEDTWLQRRLVTSGRFHHPTQSWSQQTPEFQQDSKLELVRDAILAAVNGLVSQPFKNFLGKLKPLNV
301   LNILSNCDWTFMGVVEMVILLLELFGVFWNPPDVSNFIASLLPDFHLQGPEDLARDLVPVILGGIGLAIGFTRDKVTKVMKSAVDGLRAATQLGQYGLEI
401   FSLLKKYFFGGDQTERTLKGIEAAVIDMEVLSSTSVTQLVRDKQAAKAYMNILDNEEEKARKLSAKNADPHVISSTNALISRISMARSALAKAQAEMTSR
501   MRPVVIMMCGPPGIGKTKAAEHLAKRLANEIRPGGKVGLVPREAVDHWDGYHGEEVMLWDDYGMTKILDDCNKLQAIADSAPLTLNCDRIENKGMQFVSD
601   AIVITTNAPGPAPVDFVNLGPVCRRVDFLVYCSAPEVEQIRRVSPGDTSALKDCFKLDFSHLKMELAPQGGFDNQGNTPFGKGTMKPTTINRLLIQAVAL
701   TMEKQDEFQLQGKMYDFDDDRVSAFTTMARDNGLGILSMAGLGKKLRGVTTMEGLKNALKGYKISACTIKWQAKVYSLESDGNSVNIKEERNILTQQQQS
801   VCTASVALTRLRAARAVAYASCIQSAITSILQIAGSALVVNRAVKRMFGTRTATLSLEGPPREHKCRVHMAKAAGKGPIGHDDVVEKYGLCETEEDEEVA
901   HTEIPSATMEGKNKGKNKKGRGRRNNYNAFSRRGLNDEEYEEYKKIREEKGGNYSIQEYLEDRQRYEEELAEVQAGGDGGIGETEMEIRHRVFYKSKSRK
1001  HHQEERRQLGLVTGSDIRKRKPIDWTPPKSAWADDEREVDYNEKISFEAPPTLWSRVTKFGSGWGFWVSPTVFITTTHVIPTSAKEFFGEPLTSIAIHRA
1101  GEFTLFRFSKKIRPDLTGMILEEGCPEGTVCSVLIKRDSGELLPLAVRMGAIASMRIQGRLVHGQSGMLLTGANAKGMDLGTIPGDCGAPYVYKRANDWV
1201  VCGVHAAATKSGNTVVCAVQASEGETTLEGGDKGHYAGHEIIKHGCGPALSTKTKFWKSSPEPLPPGVYEPAYLGGRDPRVTVGPSLQQVLRDQLKPFAE
1301  PRGRMPEPGLLEAAVETVTSSLEQVMDTPVPWSYSDACQSLDKTTSSGFPYHRRKNDDWNGTTFVRELGEQAAHANNMYEQAKSMKPMYTGALKDELVKP
1401  EKVYQKVKKRLLWGADLGTVVRAARAFGPFCDAIKSHTIKLPIKVGMNSIEDGPLIYAEHSKYKYHFDADYTAWDSTQNRQIMTESFSIMCRLTASPELA
1501  SVVAQDLLAPSEMDVGDYVIRVKEGLPSGFPCTSQVNSINHWLITLCALSEVTGLSPDVIQSMSYFSFYGDDEIVSTDIEFDPAKLTQVLREYGLRPTRP
1601  DKSEGPIIVRKSVDGLVFLRRTISRDAAGFQGRLDRASIERQIYWTRGPNHSDPFETLVPHQQRKVQLISLLGEASLHGEKFYRKISSKVIQEIKTGGLE
1701  MYVPGWQAMFRWMRFHDLGLWTGDRNLLPEFVNDDGV*
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ORF2

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1                                 MMMASKDAPQSADGASGAGQLVPEVNTADPLPMEPVAGPTTAVATAGQVNMIDPWIVNNFVQSPQGEF
69    TISPNNTPGDILFDLQLGPHLNPFLSHLSQMYNGWVGNMRVRILLAGNAFSAGKIIVCCVPPGFTSSSLTIAQATLFPHVIADVRTLEPIEMPLEDVRNV
169   LYHTNDNQPTMRLVCMLYTPLRTGGGSGNSDSFVVAGRVLTAPSSDFSFLFLVPPTIEQKTRAFTVPNIPLQTLSNSRFPSLIQGMILSPDASQVVQFQN
269   GRCLIDGQLLGTTPATSGQLFRVRGKINQGARTLNLTEVDGKPFMAFDSPAPVGFPDFGKCDWHMRISKTPNNTGSGDPMRSVSVQTNVQGFVPHLGSIQ
369   FDEVFNHPTGDYIGTIEWISQPSTPPGTDINLWEIPDYGSSLSQAANLAPPVFPPGFGEALVYFVSAFPGPNNRSAPNDVPCLLPQEYITHFVSEQAPTM
469   GDAALLHYVDPDTNRNLGEFKLYPGGYLTCVPNGVGAGPQQLPLNGVFLFVSWVSRFYQLKPVGTASTARGRLGVRRI*
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ORF3

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1                                                                             MAQAIIGAIAASAAGSALGAGI
23    QAGAEAALQSQRYQQDLALQRNTFEHDKDMLSYQVQASNALLAKNLNTRYSMLIAGGLSSADASRAVAGAPVTRLIDWNGTRVAAPRSSATTLRSGGFMA
123   VPMPVQPKSKTPQSSGFSNPAYDMSTVSSRTSSWVQSQNSLRSVSPFHRQALQTVWVTPPGSTSSSSVSSTPYGVFNTDRMPLFANLRR*
```

Fig. 2. The deduced amino acid sequences encoded by the three major ORFs of Southampton virus RNA (28). The GenBank accession number for the cDNA nucleotide sequence is L07418. The first codon of ORF1 occurs at nt 146. The putative helicase (GPPGIGKT) and RNA polymerase (GLPSG and YGDD) motifs are underlined. The overlapping-reading frames ORF2 and ORF3 begin at nt 5343 and 6983, respectively. Asterisks denote positions of terminator codons. Sequence analyses were performed with DNASTAR software (22).

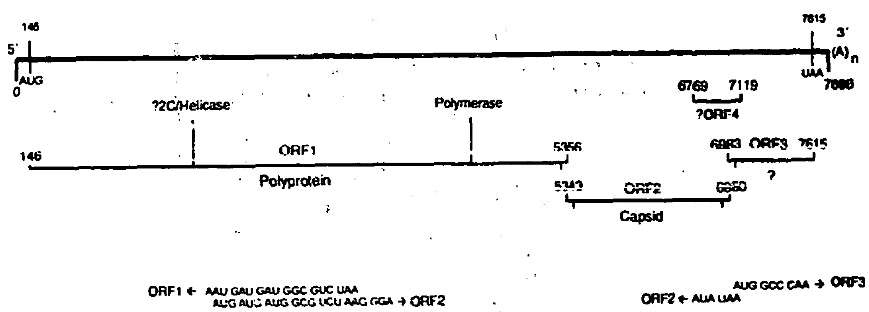

Fig. 3. Genome organization of Southampton virus. The nucleotide coordinates and the putative translation products of the ORFs are indicated. The intergenic regions showing the nucleotide sequences of the overlapping ORFs are also shown. Question marks indicate unknown or uncertain function; (A)$_n$, polyadenylate tail.

Fig. 4. Comparison of Southampton virus protein sequences with partial sequences of Norwalk virus proteins. Identical amino acid residues (28) are denoted as dots. **(A)** Alignment with the immunogenic region of Norwalk virus (16). **(B)** Alignment with the putative RNA-dependent RNA polymerase of Norwalk virus (15). Asterisks mark the conserved RNA polymerase motifs.

```
A           680       690       700       710       720       730
QGGFDNQGNTPFGKGTMKPTTINRLLIQAVALTMERQDEFQLQGKMYDFDDDRVSAFTTMARDNGLGIL
...............V..........................PT....T...A...R...A....LI

740       750       760       770       780       790
SMAGLGKKLRGVTTMEGLKNALKGYKISACTIKWQAKVYSLESDGNSVNIKEERNIL   Southampton
...S....  S...I.......S.....K.S.Q..SR..II....A..Q...DKQA.   Norwalk

B 1470      1480      1490      1500      1510      1520      1530
HFDADYTAWDSTQNRQIMTESFSIMCRLTASPELASVVAQDLLAPSEMDVGDYVIRVKEGLPSGFPCTSQ
.........................S........E....................................
                                                           *****

1540      1550      1560      1570      1580      1590
VNSINHWLITLCALSEVTGLSPDVIQSMSYFSFYGDDEIVSTDIEFDPAKLTQVLRE   Southampton
.......I........A...  .V.................D....R...I.K.   Norwalk
                                        ****
```

145 nucleotides (21), and the 3′ end contains 81 untranslated nucleotides preceding the poly(A) tail. Computer analysis (22) revealed three open reading frames (ORFs) (Fig. 2), each with the consensus translation initiator sequence RNNAUGG (23). A fourth potential ORF with a weaker translation initiator sequence (AUUAUGU) is located in frame 1 and overlaps ORF2 and ORF3 (Fig. 3). ORF1 starts at the first AUG codon [nucleotide (nt) 146] and codes for a 1737–amino acid polypeptide. Such large ORFs are characteristic of picornavirus polyproteins and have recently been found in the genomes of feline calicivirus (FCV) and rabbit hemorrhagic disease virus (RHDV) (24, 25).

Comparison of the Southampton virus polyprotein sequence with the two published partial amino acid sequences of Norwalk virus revealed 79% similarity to an immunogenic region covering amino acids 669 to 794 (Fig. 4A) and 93% similarity to a region coding for the RNA-dependent RNA polymerase (Fig. 4B). Similarity to the translated sequences of FCV (24) and RHDV (25) was limited to the polymerase region of the polyprotein and was approximately 31% and 27%, respectively. Amino acids 510 to 517 contain a nucleotide binding motif [GXXGXGK(S or T), where G is Gly, K is Lys, S is Ser, T is Thr, and X is any amino acid], which is typical of the picornavirus-encoded helicase P2C; this motif occurs in an almost identical position in the RHDV polyprotein (25).

We assigned ORF2 to the major capsid protein on the basis of its limited similarity to the capsid proteins of FCV (31%, amino acid residues 15 through 223) and RHDV (30%, amino acid residues 4 through 224). There are three potential initiator codons in ORF2, with the third most closely resembling the consensus (23). There is a 17-nt overlap between the terminator sequence of ORF1 and the first predicted codon of ORF2 (Fig. 3). A 1-nt overlap occurs at the junction of ORF2 and ORF3, a situation analogous to the 4-nt overlap found at a similar position in the FCV genome. ORF3 is of unknown function but has counterpart ORFs in the FCV and RHDV genomes. It shows limited similarity to FCV ORF3 (24%, amino acid residues 80 through 124) and is approximately double its size. ORF4 does not appear to have a counterpart in either the FCV or RHDV genomes. A similarly sized ORF (369 nt) that overlaps ORFs 1 and 2 has been identified in the 7.5-kb RNA genome of human hepatitis E virus (HEV), another virus found in stool samples. Like the two characterized animal caliciviruses (24, 25), HEV encodes a large ORF and a capsid protein (26); however, the taxonomic position of HEV remains unresolved.

The complete nucleotide sequence of Southampton virus, together with comparative biophysical and biochemical studies recently reviewed by Greenberg and Matsui (27), strongly suggest that human SRSVs are members of the Caliciviridae. The similarity in genome organization between Southampton virus and the animal caliciviruses is clear, although their ORF sizes and frameshift positions do not match precisely. Indeed, the RHDV genome appears to encode the capsid sequence within the large polyprotein ORF, whereas the FCV genome contains a stop codon and frameshift between the polyprotein and capsid gene. The Southampton virus genomic organization more closely resembles the arrangement seen in FCV, although the capsid gene appears to be smaller and ORF3 double in size. The high degree of similarity at the amino acid level between Southampton virus (serotype 3) and the partial sequences available for Norwalk virus (serotype 1) further confirms the relatedness of these two viruses.

The sequence and genome organization of SRSVs shows that the virus encodes three ORFs and supports the proposal that it is a member of the Calciviridae. The availability of such nucleotide sequence information will make it possible to develop highly sensitive diagnostic assays and will allow comprehensive molecular epidemiological and phylogenetic studies on this important group of human pathogens.

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18. The family outbreak occurred on 3 July 1991 with the 2-year-old child as the index case. Illness started with vomiting (six episodes) and elevated temperature (38°C) over a 6-hour period. The index case had only one diarrheal episode, which occurred 8 hours after the onset of vomiting; this sample was frozen at −70°C within 1 hour of collection. Both parents simultaneously exhibited symptoms of fever, nausea, vomiting, and explosive diarrhea, which started 22 hours after the first vomiting episode of the index case and lasted for 36 hours. The outbreak source was not identified.
19. Viral RNA was isolated directly from 0.5 g of stool sample (estimated to contain 10^6 virus particles per gram) by extraction with RNAzol B (Biogenesis, Bournemouth, United Kingdom). The RNA was purified from the aqueous phase by adsorption onto silica particles (RNAid, BIO 101, Vista, CA). We produced single-stranded cDNA with ribonuclease (RNase) H⁻ (minus indicates mutant) Moloney murine leukemia virus reverse transcriptase (Superscript, Gibco BRL, Paisley, United Kingdom) by priming total stool RNA with oligo$(dT)_{20}$, random hexamers, or specific primers. RNA-DNA hybrids were treated with RNase H and purified by Sephacryl S-400 chromatography before polymerase chain reaction (PCR) amplification. Amplification (Perkin-Elmer Cetus Cycler model 9600) of segments of viral cDNA for sequencing was achieved with Taq or Pfu polymerases and oligo$(dT)_{20}$ and specific primers. Extension times of 3 to 5 min were used to favor amplification of relatively long (2- to 4-kb) cDNA molecules. Successful amplification of 3.1-kb and 2.4-kb DNA fragments was achieved with the following primers: NOR3 (5′-TTGATGCAGATTATACAG-3′) (nt 4545 through 4562) versus oligo$(dT)_{20}$ and NOR9 (5′-GCTACGCAGTGTCACCAC-3′) (nt 2380 through 2397) versus SV1 (5′-ACTCTGTCATGATTTGCCTA-3′) (nt 4601 through 4582). The 3.1-kb PCR product that extended from the RNA polymerase gene to the polyadenylated 3′ terminus and the 2.4-kb PCR product that extended from the RNA polymerase gene to the upstream immunogenic region were digested with the restriction endonucleases Hae III, Rsa I, and Alu I and cloned into M13mp8 to generate a series of libraries for sequencing. Direct Taq polymerase sequencing was then performed on both strands of the PCR-amplified cDNA pool. Initially, sequencing was performed manually with Sequenase (U.S. Biochemical Corp., Cleveland, OH) and [α-^{35}S]deoxyadenosine triphosphate; the later stages of the work were completed with Taq DyeDeoxy terminator cycle sequencing on an automated sequenator (Applied Biosystems 373A).
20. To obtain sequence information for PCR amplification of the 5′ terminus of the Southampton virus genome, we used two approaches. A random-primed cDNA library of 3×10^5 recombinants from whole stool total RNA was constructed in λGEM2 (Promega, Southampton, United Kingdom). A single recombinant selected from this library carried a 2.6-kb cDNA insert that stretched from the upstream immunogenic sequence toward the 5′ terminus of the RNA (nt 21 to 2543). Subsequent sequence analysis revealed that this recombinant was truncated by 20 nt from the authentic 5′ terminus of the genome. To define the 5′ terminus of the Southampton virus genome, we used the procedure of M. A. Hofmann and D. A. Brian [*PCR Methods Appl.* 1, 43 (1991)]. Briefly, single-stranded linear cDNA was concatenated with T4 RNA ligase, which joined the 5′ terminus to the 3′ end of the primer used for the initiation of cDNA synthesis. A nested set of internal primers was then used to amplify the region generated by ligation of the 5′ and 3′ termini. Sequence analysis of the ligated junction within this fragment defined the sequence of the 5′ terminus of the genome. A primer complementary to this 5′ terminal sequence was synthesized and used to complete the PCR amplification, which allowed direct nucleotide sequencing of the 5′ terminal 2.2 kb of the Southampton virus genome. The genome analysis was concluded with specific oligonucleotide primers so that the entire sequence of Southampton virus cDNA was determined for both strands by direct PCR sequencing.
21. Computer analysis of the 5′ region of the Southampton viral RNA with the FOLD program [M. Zuker, *Nucleic Acids Res.* 9, 133 (1981)] predicted extensive secondary structures surrounding the putative AUG initiation codon of the polyprotein. This codon is preceded by 21 un-

paired nucleotides which in turn are preceded by four stem-loop structures. The AUG initiator codon itself also overlaps part of a predicted stem-loop structure.
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28. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
29. We thank M. W. Carter for excellent technical assistance and M. A. Pickett for advice on automated DNA sequencing.

25 September 1992; accepted 4 December 1992

Activation of the Sphingomyelin Signaling Pathway in Intact EL4 Cells and in a Cell-Free System by IL-1β

Shalini Mathias, Anas Younes, Chu-Cheng Kan, Irene Orlow, Cecil Joseph, Richard N. Kolesnick*

The mechanism of interleukin-1 (IL-1) signaling is unknown. Tumor necrosis factor–α uses a signal transduction pathway that involves sphingomyelin hydrolysis to ceramide and stimulation of a ceramide-activated protein kinase. In intact EL4 thymoma cells, IL-1β similarly stimulated a rapid decrease of sphingomyelin and an elevation of ceramide, and enhanced ceramide-activated protein kinase activity. This cascade was also activated by IL-1β in a cell-free system, demonstrating tight coupling to the receptor. Exogenous sphingomyelinase, but not phospholipases A_2, C, or D, in combination with phorbol ester replaced IL-1β to stimulate IL-2 secretion. Thus, IL-1β signals through the sphingomyelin pathway.

Hydrolyis of sphingomyelin to ceramide at the plasma membrane by a neutral sphingomyelinase may initiate a cascade that functions in signaling (*1*–*6*). Ceramide may stimulate a Ser-Thr kinase termed ceramide-activated protein kinase to transduce the signal (*2*–*4*). Ceramide-activated protein kinase is membrane-bound, Mg^{2+}-dependent, and defined by its capacity to phosphorylate a synthetic peptide (amino acids 663 to 681) derived from the amino acid sequence surrounding Thr^{669} of the epidermal growth factor receptor (EGFR). Ceramide-activated protein kinase may be a member of an emerging family of proline-directed Ser-Thr kinases that includes the extracellular signal-regulated (also referred to as mitogen-activated) and $p34^{cdc2}$ kinases (*7*). Substrates for these kinases contain the minimal recognition sequence, X-Ser/Thr-Pro-X, in which the phosphorylated site is flanked on its COOH-terminus by a proline residue and X can be any amino acid.

Tumor necrosis factor (TNF)–α may use the sphingomyelin pathway for signaling (*3, 4, 6*). TNF stimulates this pathway early during HL-60 cell differentiation into monocytes, and synthetic ceramide analogs bypass receptor activation and directly induce differentiation (*4*–*6*). This cascade can be reconstituted in a cell-free system comprised of extracts of HL-60 cells, which demonstrates tight coupling of this pathway to the TNF receptor (*4*). The present studies were performed because of numerous reports that TNF and IL-1 stimulate a common set of events in diverse biologic systems (*8*).

The murine thymoma EL4 cell line is a well-defined IL-1–responsive cell line that expresses functional IL-1 receptors (*9, 10*). Upon stimulation with IL-1, these cells up-regulate the IL-2 receptor and secrete IL-2 (*10*). Initial studies were designed to investigate the effects of IL-1β on cellular sphingomyelin content. Cells grown in Dulbecco's modified Eagle's (DME)–Ham's F-12 medium containing 10% horse serum and [^{3}H]choline (1 μCi/ml) were resuspended in the same medium at 10 × 10^6 cells per milliliter and stimulated with IL-1β. Under these conditions, IL-1β induced time- and concentration-dependent sphingomyelin hydrolysis (Fig. 1, A and B). A maximally effective concentration of IL-1β (40 ng/ml) induced a detectable reduction by 2 min in sphingomyelin content from a baseline of 800 ± 14 pmol per 10^6 cells (mean ± SEM) to 648 ± 16 pmol per 10^6 cells (P < 0.005) (*11*) at 30 min. Concentrations of IL-1β of 0.01 ng/ml were effective, with a maximal effect at 10 ng/ml [effective dose (ED_{50}) ~ 2 ng/ml (Fig. 1B)]. A similar reduction in sphingomyelin content after IL-1 stimulation was determined by direct measurement of phosphorus content (*12*). In contrast, the content of phosphatidylcholine, the other major choline-containing phospholipid, was unchanged.

Under the same conditions, IL-1β in-

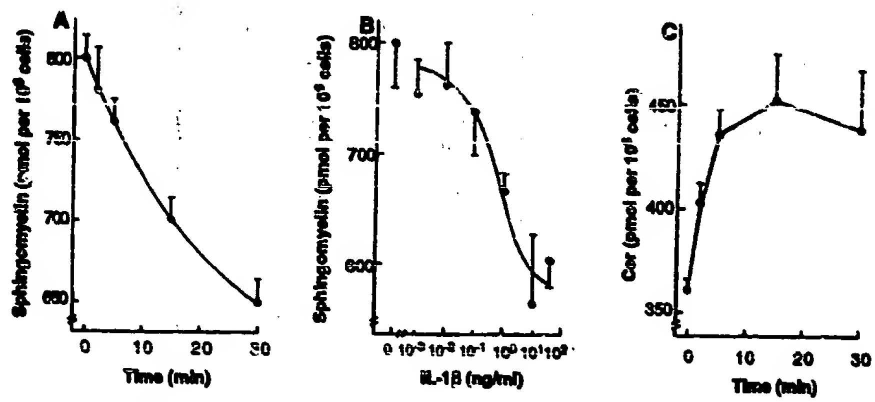

Fig. 1. IL-1β effects on sphingomyelin and ceramide in EL4 cells. (**A** and **B**) Sphingomyelin time course (A) and dose response (B). (**C**) Ceramide time course. Cells were grown to growth arrest (1 to 1.5 × 10^6 cells per milliliter) in DME–Ham's F12 medium (1:1, v/v) containing 10% horse serum. For sphingomyelin measurements, [^{3}H]choline (1 μCi/ml) was added 48 hours before an experiment. The use of [^{3}H]choline as a measure of sphingomyelin content was validated by simultaneous phospholipid phosphorus measurements (*12*). On the day of an experiment, cells were resuspended in the same medium at 10 × 10^6 cells per milliliter and stimulated with IL-1β (40 ng/ml) for the indicated times (A and C) or for 30 min with increasing concentrations of IL-1β (B). After extraction of lipids, sphingomyelin was resolved by TLC (*30*). Ceramide was quantified enzymatically with the use of the *Escherichia coli* diacylglycerol kinase reaction as described (*31*). Each value represents the mean ± SEM of triplicate determinations from four experiments in (A), one representative of four similar studies performed in triplicate in (B), and triplicate determinations from ten experiments in (C).

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  TITLE     Sequence and genome organization of a human small round-structured
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  JOURNAL   Science 259 (5094), 516-519 (1993)
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Virus Genes 10:2, 149–152, 1995

A Conserved Sequence Motif at the 5′ Terminus of the Southampton Virus Genome Is Characteristic of the Caliciviridae

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Received January 16, 1995; accepted February 13, 1995

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Abstract. We have determined the 5′ terminal cDNA sequence for the genome of Southampton virus, a recently characterized, human, small round-structured virus (SRSV). Genomic RNA was extracted directly from a stool sample and amplified by RT-PCR by homopolymer tailing of the 3′ terminus of ~~the cDNA. The additional sequence increases the overall length of the Southampton virus genome by~~ 12 nucleotides, resulting in a significant change to the genome organization by extending the first large open reading frame (ORF) by 51 amino acids. The 5′ terminal bases pGpT and the presence of conserved genome and putative subgenomic RNA terminal motifs are now prominent features shared between the human SRSV Southampton virus and the animal caliciviruses rabbit hemorrhagic disease virus and feline calicivirus.

Key words: calicivirus, Southampton virus, genome organization, 5′ terminal motif

Introduction

Animal viruses belonging to the Caliciviridae family include the prototype vesicular exanthema of swine virus (VESV), San Miguel sea lion virus (SMSV), rabbit hemorrhagic disease virus (RHDV), and feline calicivirus (FCV) (1,2). These viruses are nonenveloped and contain a positive-sense polyadenylated RNA genome of ~7.5 kb (3). Most characteristically the viruses have a distinctive cup-shaped capsid morphology when examined by electron microscopy and the capsid is comprised of a single polypeptide species of 60–73 kD (4).

The complete nucleotide sequences of RHDV (5) and FCV (6) show these virus genomes are similarly organized, with a large polyprotein located toward the 5′ end of the genome followed by an ORF of approximately 2 kb encoding the structural capsid protein; in RHDV this second ORF is fused to ORF1. A third small ORF encoding a basic protein of unknown function is located at the 3′ end of the genome. In both these viruses the capsid is additionally encoded by a subgenomic mRNA of ~2.3 kb. In VESV a VPg-like protein has been demonstrated (7) and in RHDV the genomic and subgenomic RNAs are packaged into virus particles and carry a VPg protein at their 5′ termini (8).

Calici-like viruses have long been associated with human infections, especially the Norwalk or small round structured (SRSV) group of viruses, which cause acute gastroenteritis in both adults and children (3). Molecular characterization of the SRSVs has been greatly impeded because there is no cell culture system for these viruses. However, the genome sequences for two independent SRSV isolates, the Southampton virus and the prototype Norwalk virus, have recently been obtained (9,10). These studies have relied on either the direct extraction of RNA from a single clinical specimen (Southampton virus) or

the use of purified viruses from pooled stool material from human volunteers (Norwalk virus). Sequence analysis showed both viruses contain a single-stranded positive-sense RNA genome that encodes three ORFs whose sequence and organization strongly support proposals that the SRSVs are members of the Caliciviridae.

However, a major difference in the reported genome sequences of Southampton virus and Norwalk virus was the apparent lack of conserved motifs at the 5′ ends of the putative subgenomic region and the 5′ termini of their respective genomes. Such conserved sequences are characteristic of the RHDV and FCV genomes, and may be involved in the regulation of virus replication and the packaging of RNA. In this paper we confirm the presence of similar conserved motifs in the SRSV Southampton virus, adding further support for the inclusion of the SRSVs within the Caliciviridae.

Materials and Methods

Southampton Virus

The source of virus was a single stool sample collected from the index case of a previously described family outbreak of vomiting and diarrhea that occurred in Southampton, United Kingdom in 1991 (9).

Homopolymer Tailing of cDNA

Definition of the 5′ terminal sequences of the genome was achieved using a modified method based on the RACE kit supplied by Gibco-BRL (11). Southampton virus RNA was isolated directly from 0.5 g stool sample by extraction with RNAzol B and was purified by adsorption to silica particles (12). For the amplification of the genome RNA terminus, single-stranded cDNA synthesis was primed with SV103 $5'^{302}$TTGATC AATGGGCCTGTCA$^{283}3'$. The 3′ cDNA terminus was tailed with dATP using terminal transferase according to the manufacturer's instructions. Tailed cDNA was amplified in a standard PCR reaction (13) with primers SV104 $5'^{239}$GTCGGTCCCTCTGTTGTT$^{222}3'$ and SV27 $5'^{176}$CAATTAACCCCAGAGCCAT$^{158}3'$ and T_{25}VN. The PCR product was sequenced directly and also was cloned into M13 for sequence analysis using standard techniques (14).

Primers and Sequencing

Primers for cDNA synthesis, polymerase chain reaction (PCR), and sequencing were synthesized using a Millipore 8909 Expedite synthesizer using β cyanoethyl phosphoramidite chemistry. Polymerase chain reaction amplicons for cloning and sequencing were purified using Wizard PCR purification columns (Promega, UK). Sequence data were collected using an Applied Biosystems 373A automated sequencer.

Results

To define the 5′ terminus of the Southampton virus genomic RNA by the technique of rapid amplification of cDNA ends (11), primer SV103 was used to prime cDNA synthesis and the cDNA was polyadenylated with terminal transferase. Successful PCR amplification with T_{25}VN and the genome-specific primers was achieved (data not shown). Direct PCR sequencing of these fragments and sequence analysis of the M13 clones showed the PCR products were 12 nucleotides longer than the previously defined 5′ terminus of the Southampton virus (9). Interestingly, these extra 12 bases, when added to the original sequence and translated, revealed an identical amino acid sequence motif (MMMASKD) to that predicted at the start of the capsid protein and extended the predicted polyprotein open reading frame a further 51 amino acids from the original initiation codon, at nucleotide 146, to a new initiation codon at nucleotide 5. In addition, immediately prior to the new start codon is an in-frame stop codon at nucleotide position 2. These data are summarized in Fig. 1.

Discussion

Comparison of the published 5′ terminal sequences of both the Southampton and Norwalk virus genomes (9,10) revealed only limited partial sequence homology with an internal sequence

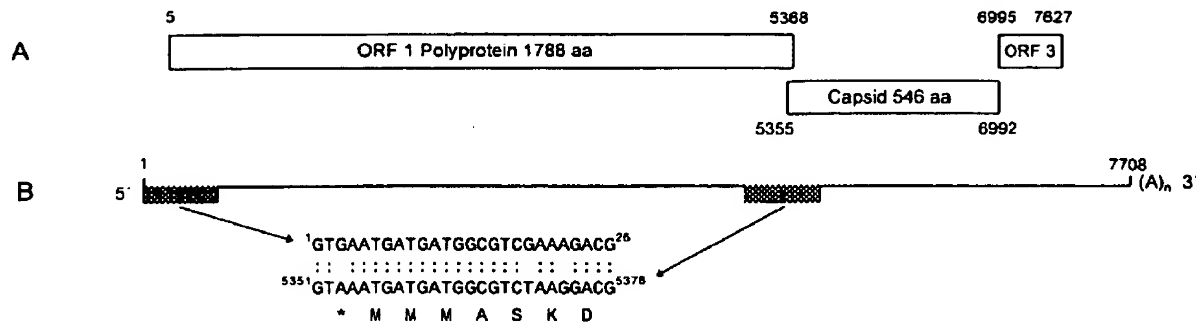

Fig. 1. Genome organization of Southampton virus. *A:* The 7708 nucleotide SV genome (GenBank accession no. L07418) encodes three large ORFs represented by the open boxes. The nucleotide coordinates define the first and last nucleotides of the predicted translation products. *B:* Schematic showing the relative position and alignment of the repeated motifs (shaded boxes).

close to the putative capsid initiator codon. Thus, if these two viruses are genuine members of the Caliciviridae, this analysis strongly suggested that both these virus genome sequences may be incomplete. It was therefore of interest to use an alternative technique in a further attempt to define precisely the 5′ terminus of the Southampton virus genomic RNA. Computer predictions suggested the 5′ terminal regions of both the Southampton virus and the Norwalk virus contain extensive secondary structures (9,10). The 5′ genome terminus of the Southampton virus was originally defined using the method of Hoffmann and Brian (15), in which single-stranded cDNA is concatenated with T_4 RNA ligase and the unknown 5′ terminus is amplified by PCR with flanking primers. In this method premature reverse transcriptase pause points, due to secondary structure features, would also amplify in the PCR, with the consequence that the sequence of longer, low abundance authentic transcripts would be unlikely to be detected in the cDNA pool. For the Norwalk virus, other techniques were unable to extend the 5′ terminal sequence obtained from the consensus sequence of the longest cDNA clones (10). The Norwalk virus 5′ terminal genome sequence designated from these clones was in agreement with the 5′ genome terminus of the Southampton virus, although the method used to generate double-stranded cDNA for the Norwalk virus (16) was most likely to result in clones that were truncated at their 5′ termini.

The additional nucleotide sequence determined for the 5′ terminus of the Southampton virus using the RACE technique is highly significant and dramatically alters the genome map (Fig. 1). The 5′ terminal bases of pGpT are now present in the Southampton virus genome and are also conserved in RHDV and FCV. Also, in both RHDV and FCV the 5′ terminal sequences of the genomic RNA show a high degree of similarity with the 5′ terminal sequence of their respective subgenomic RNAs around the putative capsid initiator codons. The equivalent motif in Southampton virus is now clearly present and is repeated internally within the genome between nucleotides 5351 and 5376. Figure 1 shows there are 23 identical residues within this 26 nucleotide motif. Two of the three base changes are silent mutations within the predicted amino-terminal coding regions of ORF1 and ORF2. The other nucleotide substitution is immediately upstream of the initiator methionine. This is also a silent mutation that preserves the termination codon situated at this position. The exact function of this motif is unknown, but it may play a role in replication or packaging of viral RNA.

Nothing is known about translational initiation in the caliciviruses. The presence of a 5′ terminal VPg protein in FCV and RHDV is suggestive of an IRES-like mechanism (17), although others (10) were unable to find polypyrimidine tracts in the sequence of the Norwalk virus close to the genome terminus to support this hypothesis. As there is no cell culture system for the SRSVs, the next important step in the study of SRSV biology is the development of an authentic genome-length cDNA that can be used to produce RNA in vitro for transfection of tissue culture

cells and production of infectious virus. If the conserved terminal sequence motifs are important in SRSV genome replication, then inclusion of this motif will be essential for producing an authentic "full-length" recombinant cDNA for these studies.

Acknowledgments

This work was supported by the Wellcome Trust. BLL is the recipient of a University of Southampton research scholarship.

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PMID: 3020085 [PubMed - indexed for MEDLINE]

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JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1986, p. 456–459
0095-1137/86/090456-04$02.00/0

Vol. 24, No.3

Enzyme-Linked Immunosorbent Assays for Snow Mountain and Norwalk Agents of Viral Gastroenteritis

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Received 30 January 1986/Accepted 19 May 1986

Enzyme-linked immunosorbent assays (ELISAs) for antigen detection and blocking ELISAs for serum antibody rises were developed for the Snow Mountain and Norwalk agents of viral gastroenteritis. The ELISAs were as sensitive as the existing radioimmunoassays and were specific for the Snow Mountain or Norwalk agent. The blocking ELISAs detected the same number of significant rises in antibodies to these agents as did the existing blocking radioimmunoassays.

The Snow Mountain agent (SMA) is a 27- to 32-nm virus that is morphologically similar to but antigenically distinct from the Norwalk agent (5, 7, 19). SMA was the etiologic agent of a large outbreak of gastroenteritis at a resort in Granby, Colo. (22).

The development of a radioimmunoassay (RIA) for the Norwalk virus by Greenberg et al. (14) facilitated large-scale epidemiologic studies of this agent (11, 12, 14, 20). We subsequently developed a similar solid-phase microtiter RIA for SMA antigen detection and a blocking RIA for antibody titration with the stools and sera from the SMA volunteer study (8). This report describes the conversion of our existing SMA and Norwalk agent RIAs to the enzyme-linked immunosorbent assay (ELISA) format by using the biotin-avidin system (10, 15).

Sera and stool specimens were obtained from individuals involved in the SMA outbreak in Granby, Colo. (22), and from the subsequent study of volunteers challenged with SMA (7). Stools containing the Norwalk agent and sera were from a study of volunteers challenged with the Norwalk agent (6). Stools containing rotavirus were from children with acute gastroenteritis in Burlington, Vt. Feline calicivirus (Bolin strain FPL; ATCC VR652) was from the American Type Culture Collection, Rockville, Md. Coxsackie A9, coxsackie B1, echovirus 9, and poliovirus 1 (LSC strain) were obtained from R. Dolin, Rochester, N.Y. Crude 2% (wt/vol) stool homogenates were made up in 0.5% bovine serum albumin-veal infusion broth. The RIAs for the SMA and Norwalk agent were adapted from Greenberg et al. (14) and performed as described previously (8).

For the ELISAs, serum obtained from a volunteer challenged with the SMA or Norwalk agent was used to purify the immunoglobulin G (IgG) indicator antibody by ammonium sulfate precipitation and DEAE-cellulose chromatography, as described previously (14). The purified IgG was biotinylated with biotin-*N*-hydroxysuccinimide ester (Pierce Chemical Co., Rockford, Ill.) as described by Jackson et al. (18).

The ELISAs for antigen detection were performed as follows. Only the 60 inner wells of polystyrene 96-well tissue culture plates (no. 3596; Costar, Cambridge, Mass.) were used. Sera from volunteers at prechallenge and 2 weeks postchallenge with the SMA or Norwalk agent (capture antibody) were diluted 1/5,000 in phosphate-buffered saline (PBS). Samples (100 µl) of each serum were added to duplicate wells, and the plates were incubated for 6 h at 20 to 22°C. The plates were rinsed two times in washing buffer (0.5 M NaCl, PBS, 0.05% Tween 20), and the wells were filled with blocking buffer (0.5 M NaCl, PBS, 0.5% [wt/vol] gelatin, 1% fetal bovine serum). The plates were then incubated for 16 h at 4°C. The wells were rinsed two times with washing buffer, and 25 µl of 100% fetal bovine serum was added to all wells. For the test wells, 25 µl of a stool homogenate was added to each of the duplicate pre- and postchallenge serum wells. For the control wells, 25 µl of a stool homogenate (positive control, antigen present) or dilution buffer (negative control, antigen absent; 0.5 M NaCl, PBS, 0.5% [wt/vol] gelatin, 10% fetal bovine serum) was added to the duplicate pre- and postchallenge serum wells. The plates were incubated for 2 h at 37°C, excess antigen was suctioned off, and the plates were rinsed six times with washing buffer. The biotinylated IgG (indicator antibody) was diluted 1/800 in dilution buffer, and 50-µl portions were added to all wells. The plates were then incubated for 2 h at 37°C. The plates were rinsed six times with washing buffer, and 50 µl of avidin-horseradish peroxidase (Vector Laboratories, Burlingame, Calif.), diluted 1/4,000 in dilution buffer, was added per well. The plates were incubated for 30 min at 37°C and then rinsed six times with washing buffer, and 100 µl of a freshly made, 3,3',5,5'-tetramethylbenzidine (TMB; Aldrich Chemical Co., Inc., Milwaukee, Wis.) reaction mixture (0.1 mg of TMB per ml, 0.1 M citrate buffer [pH 5.0], 0.006% hydrogen peroxide) was added to all wells. The plates were incubated for 30 min at 20 to 22°C in the dark. To stop the reaction, 50 µl of 1 N HCl was added per well, and the plates were read visually or at 450 nm in the Microelisa Minireader MR590 (Dynatech Instruments, Santa Monica, Calif.). The reader was blanked with wells containing the TMB reaction mixture alone. A positive/negative (P/N) ratio of ≥2 was considered positive. This value represented the optical density at 450 nm (OD_{450}) of the duplicate postcapture antibody wells divided by the OD_{450} of the duplicate precapture antibody wells for each test specimen after the OD_{450} for the negative control wells had been subtracted. Borderline positives (P/N ratios of ≥2 to 3) were

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TABLE 1. Detection of SMA and Norwalk agent antigens by RIA and ELISA[a]

Antigen	No. of specimens with indicated reaction			
	RIA positive		RIA negative	
	ELISA positive	ELISA negative	ELISA positive	ELISA negative
SMA	12	0	0	45
Norwalk agent	5	1	1	12

[a] Stool specimens from volunteers challenged with the SMA (7) or Norwalk agent (6) were assayed by RIA and the ELISA as described in the text. For both assays, a P/N ratio of ≥2 was considered positive.

repeated and had to be repeat positive. The OD_{450} of positives had to have been at least 0.1 OD unit.

The blocking ELISAs for serum antibody were performed as follows. The test wells were coated with serum (capture antibody), blocking buffer, and SMA or Norwalk antigen, as described for the ELISA for antigen detection, except that only postchallenge (positive) serum was used as capture antibody and the antigen-positive stool homogenate was prepared in dilution buffer to give a P/N ratio of ≥4. Negative control (buffer, antigen absent) wells of postchallenge capture antibody were included.

For the blocking antibody step, excess antigen was removed from all wells by rinsing six times with washing buffer. For the test wells, a pair(s) of sera for which the titers were to be determined was diluted 1/100 in dilution buffer. Duplicate samples (100 μl) were added to the appropriate wells, followed by serial twofold dilutions of 50-μl samples into wells containing 50 μl of dilution buffer. Unblocked antigen wells (buffer only) and negative control wells (buffer, antigen absent) were included. The plates were incubated for 2 h at 37°C and rinsed six times with washing buffer. The addition of biotinylated IgG (indicator antibody), avidin-horseradish peroxidase, and TMB reaction mixture was as described for the ELISA for antigen. The endpoint was defined as the highest dilution of serum that reduced the OD_{450} of the wells by 40% in comparison with that of the unblocked antigen wells (100% = OD_{450} of unblocked antigen wells minus OD_{450} of the negative control wells; this value ranged from 0.6 to 1.2 OD_{450} units).

The substrate TMB was previously used by Beards et al. (2) and was not found to be carcinogenic in rats or positive in the Ames test (17).

To develop the ELISA for detection of SMA antigen, stool specimens from the SMA volunteer study previously described (7) that had already been tested by RIA for the presence of SMA antigen, were retested by ELISA for SMA antigen. The ELISA detected the same number of positive and negative specimens as did the RIA (Table 1). Of the 12 positive stools, 7 had initial P/N values of ≥2 to 3 and repeated positive. Of the 45 negative stools, 3 were initially low positive and repeated negative. The specificity of the ELISA for SMA is depicted in Table 2 in which it is shown that specimens containing Norwalk virus, human rotavirus, feline calicivirus, and several enteroviruses did not react in the assay.

The rises in serum antibody to the SMA detected by the ELISA were compared with rises detected by RIA in the volunteers challenged with the SMA (Table 3). All significant rises (fourfold or greater) detected by RIA were also detected by the ELISA. The magnitudes of the rises detected by each method were generally similar, although variation

TABLE 2. Specificity of SMA and Norwalk agent ELISAs

Agent[a]	No. of specimens negative in ELISA[b]:	
	SMA	Norwalk agent
Norwalk	7	
SMA		4
Human rotavirus	2	2
Feline calicivirus	1	1
Coxsackie A9	1	1
Coxsackie B1	1	1
Echovirus 9	1	1
Poliovirus 1	1	1

[a] P/N ratios for stools containing the Norwalk agent, as measured by the Norwalk agent RIA, were 4 to 11, and P/N ratios for stools containing the SMA, as measured by the SMA RIA, were 10 to 13. Stools containing rotavirus were strongly positive by Rotazyme (Abbott Laboratories, Diagnositics Div., North Chicago, Ill.). The feline calicivirus titer was 10^8 PFU/ml; the coxsackie A9, coxsackie B1, and echovirus 9 titers were 10^5 50% tissue culture infective doses per ml; and the poliovirus 1 titer was 10^9 50% tissue culture infective doses per ml.

[b] The ELISAs were performed as described in the text. None of the specimens tested was positive.

between individuals was noted. The endpoint titers were also similar in both tests.

We subsequently converted our RIA for the Norwalk agent to the same ELISA format described for the SMA. Sera and stool specimens from volunteers challenged with the Norwalk agent (6) were used in these assays. The sensitivity of the ELISA for the Norwalk antigen in stool specimens was similar to that of the RIA (Table 1). Of the six positive stools, only one low-positive stool (P/N ratio of ≥2 to 3) was repeat positive, and none of the negative stools was initially positive. The Norwalk ELISA was specific for Norwalk antigen; it did not detect SMA, human rotavirus, feline calicivirus, or enterovirus antigens (Table 2).

The blocking RIA for Norwalk antibody was also converted to the ELISA format. The numbers of significant antibody rises in paired sera from eight volunteers challenged with the Norwalk agent were the same (six of eight) in both assays, and the extents of the rises and endpoint titers of the sera were also similar (Table 4).

In summary, we converted existing RIAs for the detection of the SMA and Norwalk agents to biotin-avidin ELISAs but

TABLE 3. Comparison of blocking assay for SMA antibody by RIA and ELISA[a]

Volunteer	RIA titer			ELISA titer		
	S_1	S_2	Rise (fold)	S_1	S_2	Rise (fold)
1	100	6,400	64	<100	3,200	64
2	200	800	4	<100	800	16
3	100	800	8	100	400	4
4	3,200	1,600	−2	200	400	2
5	100	1,600	16	<100	800	16
6	<100	200	4	<100	3,200	64
7	200	200	0	200	200	0
8	<100	1,600	32	<100	6,400	128
9	<100	800	16	<100	800	16
10	100	3,200	32	<100	400	8
11	<100	<100	0	<100	<100	0
12	200	200	0	200	200	0

[a] As described previously (7), pre- and 3-week-postchallenge sera (S_1 and S_2, respectively) from volunteers previously examined by blocking RIA were tested by blocking ELISA as described in the text. The endpoint titer was the highest dilution of serum that reduced the 100% OD_{450} readings by ≤40%.

TABLE 4. Comparison of blocking assay for Norwalk agent antibody by RIA and ELISA[a]

Volunteer	RIA titer			ELISA titer		
	S_1	S_2	Rise (fold)	S_1	S_2	Rise (fold)
1	200	12,800	64	100	25,600	256
2	<100	12,800	256	<100	6,400	128
3	400	12,800	32	100	6,400	64
4	200	6,400	32	<100	3,200	64
5	3,200	12,800	4	800	25,600	32
6	<100	<100	0	<100	<100	0
7	1,600	1,600	0	200	200	0
8	<100	200	4	<100	200	4

[a] Volunteers were challenged with the Norwalk agent as described previously (6). Pre- and 3-week-postchallenge sera (S_1 and S_2, respectively) from volunteers previously examined by blockiing RIA were tested by blocking ELISA as described in the text. The endpoint titer was the highest dilution of serum that reduced the 100% OD_{450} readings by ≤40%.

maintained the sensitivity of the RIAs. Gary et al. (9) previously developed a biotin-avidin ELISA for Norwalk agent detection and also observed a sensitivity similar to or greater than that of their Norwalk RIA. Our ELISAs are specific for the SMA or Norwalk agent and do not cross-react with human rotavirus, feline calicivirus, or several enteroviruses. Gary et al. (9) also indicated a lack of cross-reaction between their Norwalk ELISA and stools from volunteers inoculated with the SMA. These observations help to confirm the antigenic distinctiveness of the two agents (5, 7). Herrmann et al. (16) converted their RIA for Norwalk antigen detection to an ELISA by directly linking the indicator IgG to peroxidase. This ELISA was more sensitive for antigen detection than was the RIA and did not react with stools from volunteers inoculated with the antigenically distinct Hawaii agent (5, 19).

Although the Norwalk agent (13) and SMA (21), like the caliciviruses (24), possess a single virion structural protein, our SMA and Norwalk ELISAs did not cross-react with feline caliciviruses. In an earlier study, human calicivirus-like particles did not appear to be serologically related to the Norwalk virus (13).

The blocking ELISAs to SMA and Norwalk antibody detected a number of significant antibody rises in volunteers challenged with those agents equal to those detected by the RIAs. Gary et al. (9) previously converted their Norwalk blocking RIA to an ELISA.

Norwalk virus, the prototype for the Norwalk-like viruses (1, 4, 5, 23, 25), plays a significant role in the etiology of acute gastroenteritis; 34 to 42% of the outbreaks of acute infectious nonbacterial gastroenteritis have been attributed to Norwalk virus (12, 20). Similar extensive seroepidemiologic studies with the SMA have not been performed. In addition to the original outbreak in Granby, Colo. (22), the SMA has been linked to a small outbreak in Chittenden County, Vt., by RIA serology (3). A more recent outbreak in Rochester, N.Y., associated with the ingestion of clams, has been attributed to the SMA by ELISA serology and antigen detection (B. I. Truman, H. P. Madore, M. Menegus, J. L. Nitzkin, and R. Dolin, Annu. Epidemic Intelligence Serv. Conf., p. 54, 1985). With the SMA ELISA, we can now define more clearly the role of the SMA in outbreaks of gastroenteritis.

We thank Grace Ann Duttweiler for her excellent technical assistance.

This work was supported, in part, by Public Health Service grant R22 AI20070 from the National Institutes of Health.

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Volume 12
Number 51
19 December 2002

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Norovirus (Norwalk-like virus) increase in 2002

Three thousand and twenty-nine laboratory confirmed reports of norovirus (Norwalk-like virus) infection were reported to the PHLS Communicable Disease Surveillance Centre (CDSC) in the first ten months of 2002. This represents a substantial increase from preceding years. The previous peak in reporting was in 1996, when 2437 cases were reported for the whole 12-month period. In 2002 reports did not decline during summer months as they typically have in precious years. The bulk of the recent increase was in the elderly. Sixty-eight per cent of all cases reported in the first ten months of 2002 were aged 65 years or above. Laboratory reporting of norovirus-positive results provides a timely source of trend data although noroviruses are substantially underreported by this method of surveillance because not all patients seek medical care, and of those who do, only a minority have relevant tests.

The rise in reports does not necessarily mean that the incidence has increased. Factors potentially influencing the number of reports include increased ascertainment due to awareness and improvements in diagnostic capability. A commercial ELISA for the detection of noroviruses in faecal samples is now being marketed (Dako Cytomation, Ely, UK) so testing is more readily available. Guidance on the ELISA as a preliminary screening tool for outbreaks of gastroenteritis will be published shortly (1).

The impact of noroviruses on healthcare facilities is increasingly recognised both in the medical and popular press (2-4). Since 1992, over three-quarters of norovirus outbreaks reported to the PHLS Communicable Disease Surveillance Centre occurred in either hospitals or residential homes (5). Guidance on the control of hospital outbreaks of viral gastroenteritis has been issued (6). Understanding of the epidemiology, in particular the incidence and impact of norovirus outbreaks in healthcare institutions, is, however, incomplete. For this reason, a collaborative team including the Avon Heath Protection Unit, Bristol Public Health Laboratory, and CDSC Gastrointestinal Diseases Division have introduced active, enhanced surveillance in hospitals and nursing homes (7).

Noroviruses are the group of viruses formerly known as Norwalk-like viruses (NLV) or small round structured viruses (SRSV) (8,9). These viruses have long been associated with outbreaks of a relatively mild and short-lived form of gastroenteritis, often referred to as "winter vomiting disease". A prominent symptom is projectile vomiting that can lead to widespread environmental contamination, resulting in indirect but rapid person-to-person spread.

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Outbreaks of Norwalk-like virus infection
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London public health investigation of *Salmonella* Enteritidis in raw shell eggs

In response to the recent outbreaks and increased incidence of *Salmonella enterica* serotype Enteritidis thought to be associated with raw shell eggs (1), the London local authorities' environmental health departments within the London Food Co-ordinating Group have sampled eggs from large catering outlets, hospitals, and suppliers/distributors. Samples are being examined for *Salmonella spp.* by the PHLS London Food Water and Environmental Microbiology Laboratory and isolates sent to the PHLS Laboratory of Enteric Pathogens (LEP) for confirmation and typing. Further to the results of investigations reported in *CDR Weekly* of 12 December 2002 (2), the following additional results are now available (table 1).

Table 1 *Salmonella* Enteritidis in eggs: contamination rates based on pooled samples of six eggs (Samples tested up to 9 December 2002)

Total number of eggs	Total number of samples	Origin	Lion marked	Salmonella detected
1926	321	Britain/UK	Yes	Not detected
120	20	Britain/UK	Not Known	One sample positive for *S.* Enteritidis PT4
330	55	Not known	No	One sample positive for *S.* Enteritidis PT4
480	80	Britain/UK	No	Not detected

Between the 27 November and 9 December 2002, 25 batches of eggs, comprising 476 pooled samples of six whole eggs (2856 eggs in total) in the pan London investigation. Salmonellas have been recovered from two (0.4%) of the 476 pools. Notably, no salmonellas were isolated from ten of the 25 batches of eggs investigated. Twenty-two of the batches tested were labelled as of British or United Kingdom origin. Seventeen of these batches, (321samples, 1926 eggs) were labelled of British/UK origin with the Lion Quality mark, and no salmonellae were recovered from any of these samples. *Salmonella enterica* serotype Enteritidis phage type (PT) PT4 was recovered from eggs sampled from hospital kitchens, one of three batches of non-Lion quality British/UK eggs (55 samples, 330 eggs) (1.8%) and one batch (20 samples, 120 eggs) where the details are not known. In these circumstances hospitals may wish to

consider not using raw shell eggs.

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Glycopeptide-intermediate *Staphylococcus aureus* (GISA)

The first known patient death in the United Kinigdom (UK) following an infection with vancomycin – intermediate resistant *Staphylococcus aureus* has been reported in Monklands Hospital, Lanarkshire (1). Glycopepride Intermediate resistant *S. aureus* (GISA) was first described in Japan (2) and has increased resistance to both of the glycopeptide antibiotics – vancomycin and teicoplanin – that are used for the empirical treatment of suspected infections caused by methicillin-resistant Staphylococcus aureus (MRSA).

GISA is very rare, though MRSA with resistance only to teicoplanin is encountered more frequently. In the UK, GISAs were first reported in Scotland (3) although there have been a few subsequent reports, and none have been found among over 40000 MRSA isolates submitted to the PHLS Laboratory of Hospital Infection (LHI) for typing during the past three to four years. The genetic and phenotypic mechanisms that confer the GISA phenotypes are not fully understood (nor those that confer resistance only to teicoplanin. They do not contain the vanA resistance genes described recently in MRSA isolates with higher levels of glycopeptide resistance (4).

In the recent GISA report from Scotland, the patient contracted MRSA while on the intensive therapy unit and, despite aggressive and appropriate antibiotic therapy, the MRSA persisted and eventually developed resistance to glycopeptide antibiotics (vancomycin MIC 8 mg/L). Post-mortem findings confirmed tricuspid endocarditis with metastatic lung abscesses. Ongoing investigations are exploring the possibility of cross infection, although there has been little evidence of this so far (5). The unit was closed, deep-cleaned, and screened before re-opening.

The laboratory detection of GISA is difficult since disc diffusion tests are unsatisfactory. Suspicions of such infections should be in aroused when patients infected with *S. aureus* (usually MRSA) infections do not responding clinically to glycopeptides (2). Isolates should be tested using inocula matched to the opacity of a 2 McFarland on brain heart infusion agar, with either vancomycin (and teicoplanin) E-test strips. GISA isolates are those requiring vancomycin minimum inhibitory concentrations (MICs) of 8-16 mg/L and are invariably cross-resistant to teicoplanin. Screening can also be performed by a breakpoint dilution method using 5 mg/L vancomycin incorporated into Mueller Hinton agar or 6 mg/L vancomycin in brain heart infusion agar (6). The Antibiotic Resistance Monitoring and Reference Laboratory and the Laboratory of Healthcare Associated Infection, at the PHLS Central Public Health Laboratory would be happy to receive any suspicious isolates isolated from patients in England or Wales for MIC confirmation and typing.

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Enhanced surveillance of invasive group A streptococcal infections

A two-year enhanced surveillance of invasive group A streptococcal infections in the United Kingdom (UK) will commence on 1 January 2003 as part of an important European surveillance programme (Strep-EURO) (1). The UK component of this programme will be led by the PHLS Respiratory and Systemic Infection Laboratory (Streptococcus and Diphtheria Reference Unit, [SDRU]) in collaboration with the Healthcare Associated Infections and Antimicrobial Resistance Division of the Communicable Disease Surveillance Centre.

Cases will be ascertained in two ways: by isolate submissions to SDRU, and by routine laboratory reports made to CDSC. Laboratories will be sent a short questionnaire requesting basic isolate and patient information. Microbiologists are requested to submit all isolates from cases of severe group A streptococcal infection, for typing. Severe infection is indicated by the isolation of group A Streptococcus from a site that is normally sterile (blood, cerebrospinal fluid, joint aspirates, abscesses, pericardial/peritoneal fluid, deep tissue at operation or necropsy, and bone).

This surveillance forms an essential component of the pan-European surveillance programme supported by the European Commission, Fifth Framework Programme (QLK2.CT-2002-01398) on 'Severe *Streptococcus pyogenes* invasive disease in Europe'. The project will provide a unique opportunity to measure and compare the overall burden of these infections, as well as serotype distributions, antimicrobial susceptibility patterns, and clinical manifestations among participating countries. A one day symposium entitled *Group A streptococcal infections; clinical, epidemiological and microbiological aspects* is planned for 15 May 2003, at the Central Public Health Laboratory – all contributions are welcome. Further information on this symposium will be available at the beginning of the year.

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Bacteraemia

Last updated: 19 December 2002
Next update due: 16 January 2003

PDF

Staphylococcus aureus bacteraemia: England, Wales, and Northern Ireland, July to September 2002

Key points

- Laboratories in England reported 2950 *Staphylococcus aureus* bacteraemia isolates through the voluntary reporting scheme and 4713 isolates through the mandatory reporting scheme between July and September 2002.
- There have been 172 and 105 voluntary reports of *S. aureus* bacteraemia isolates from laboratories in Wales and Northern Ireland for the same time period.
- This is the first time that data for Northern Ireland have been included in the report.
- Overall, 91% of voluntary reports contained information on susceptibility to methicillin. This is similar to the level (93%) in the previous report (1).
- In England, 42% of *S. aureus* bacteraemias were due to MRSA under the voluntary scheme and 39% MRSA attributable to the mandatory scheme.
- Methicillin resistance in Wales and Northern Ireland (voluntary reporting) was noted in 47% and 38% of *S. aureus* bacteraemia reports respectively.

This report covers *Staphylococcus aureus* bacteraemias over the third quarter of 2002 (July to September) under the voluntary (routine *CDR Weekly* reporting by laboratories) and mandatory bacteraemia reporting schemes. These bacteria were isolated from blood cultures with or without cerebrospinal fluid, by laboratories across England, Wales, and Northern Ireland. This is the first *CDR Weekly* report to include data on *S. aureus* bacteraemias from Northern Ireland. Wales and Northern Ireland do not participate in the Department of Health's (DoH) mandatory *S. aureus* surveillance scheme. The DoH, Social Services, and Public Safety (DHSSPS) in Northern Ireland have made reporting mandatory through a similar scheme.

Rates were calculated using 2000 resident population denominators for each region. Regional analyses were performed using the English regional boundaries introduced in April 2002.

Reporting of *S. aureus* bacteraemias

In the three month period between July and September 2002, 3227 reports of *S. aureus* bacteraemia were received in England (2950), Wales (172), and Northern Ireland (105) through the voluntary reporting scheme (table 1 and figure 1). Under the mandatory surveillance scheme, there were 4713 *S. aureus* bacteraemia reports in England. Among the English regions, the South East (471) had the highest number of reports under the voluntary scheme and London (947) had the highest number of reports under the mandatory scheme. The least number of reports under both schemes were received from the North East (166 for the voluntary and 229 for the mandatory scheme). Regional variations in the total number of reports received between the two reporting schemes were observed. London had the greatest discrepancy in the number of reports (592), and the West Midlands the least (37).

The overall reporting rate of *S. aureus* bacteraemia for England, Wales, and Northern Ireland was 5.9 per 100,000 for the three month period, based on voluntary reporting (figure 2). Northern Ireland had the highest

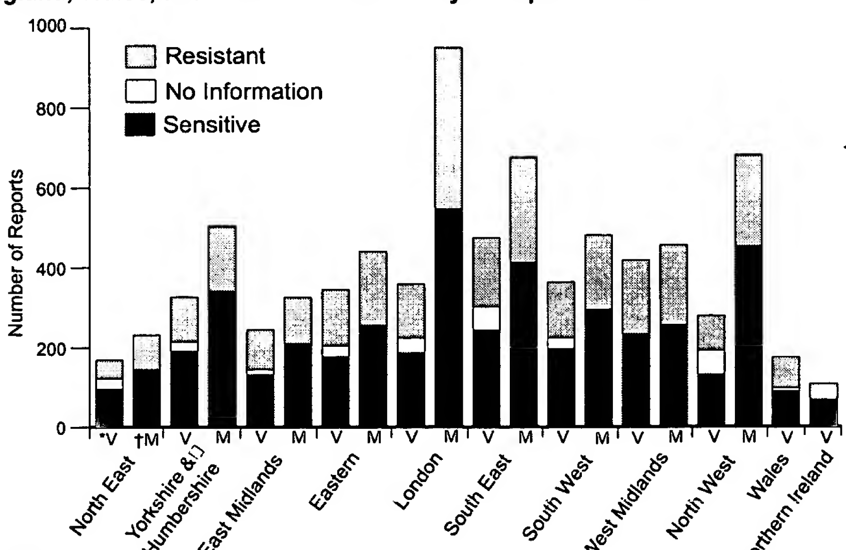
rate (6.2 per 100,000 population) followed by England (5.9 per 100,000 population) ,and Wales (5.8 per 100,000 population). Reporting rates within England ranged from 4.0 per 100,000 population in the North West to 7.8 per 100,000 in the West Midlands.

Table 1 *Staphyl c ccus aureus* bact raemia rep rts and methicillin susceptibility data*, England, Wales, and Northern Ireland: July to September 2002

Region	Reporting scheme	Resistant	(%#)	Sensitive	No information	(%)	Total
North East	Voluntary	46	33%	93	27	16%	**166**
	Mandatory	86	38%	143	0	0%	**229**
Yorkshire & Humberside	Voluntary	112	37%	188	24	7%	**324**
	Mandatory	163	33%	338	0	0%	**501**
East Midlands	Voluntary	100	44%	129	13	5%	**242**
	Mandatory	114	35%	208	0	0%	**322**
Eastern	Voluntary	139	44%	175	27	8%	**341**
	Mandatory	185	42%	252	0	0%	**437**
London	Voluntary	134	42%	184	37	10%	**355**
	Mandatory	404	43%	543	0	0%	**947**
South East	Voluntary	173	42%	240	58	12%	**471**
	Mandatory	262	39%	409	0	0%	**671**
South West	Voluntary	139	42%	194	27	8%	**360**
	Mandatory	186	39%	292	0	0%	**478**
West Midlands	Voluntary	185	45%	230	0	0%	**415**
	Mandatory	200	44%	252	0	0%	**452**
North West	Voluntary	86	40%	129	61	22%	**276**
	Mandatory	227	34%	449	0	0%	**676**
England	Voluntary	1114	42%	1562	274	9%	**2950**
	Mandatory	1827	39%	2886	0	0%	**4713**
Wales†	Voluntary	77	47%	86	9	5%	**172**
Northern Ireland†	Voluntary	40	38%	64	1	1%	**105**
England, Wales & NI	**Voluntary**	**1231**	**42%**	**1712**	**284**	**9%**	**3227**

* provisional data; #R as a percentage of R+S
† Wales & Northern Ireland do not take part in the DoH mandatory surveillance scheme

Figure 1 *Staphylococcus aureus* bacteraemia reports and methicillin susceptibility data, England, Wales, and Northern Ireland: July to September 2002

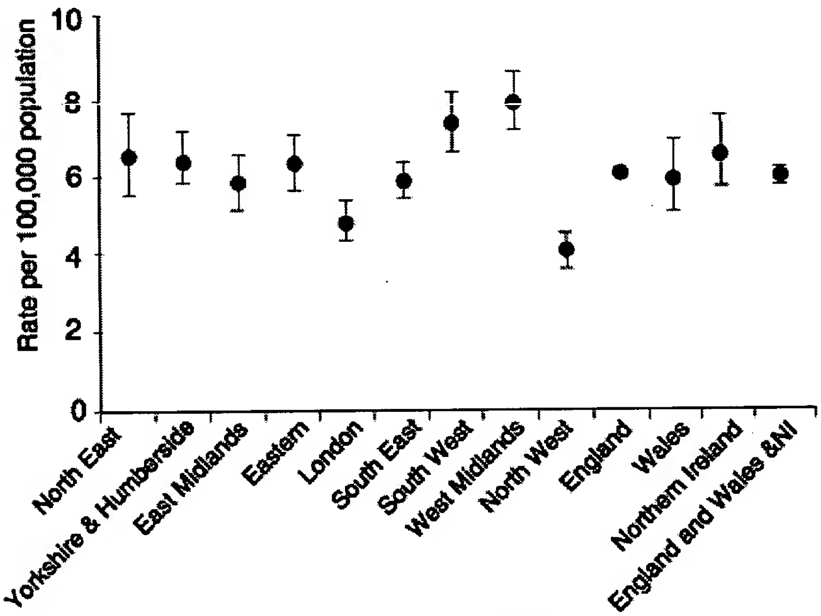


*V = Voluntary
†M = Mandatory Wales and Northern Ireland do not take part in the DoH mandatory surveillance scheme
Wales and Nothern Ireland do not take part in the DoH mandatory surveillance scheme

Table 2 ***Staphylococcus aureus*** **bacteraemia r p rts (v luntary reporting) and susceptibility data, England, Wales, and Northern Ireland: July t September 2002**

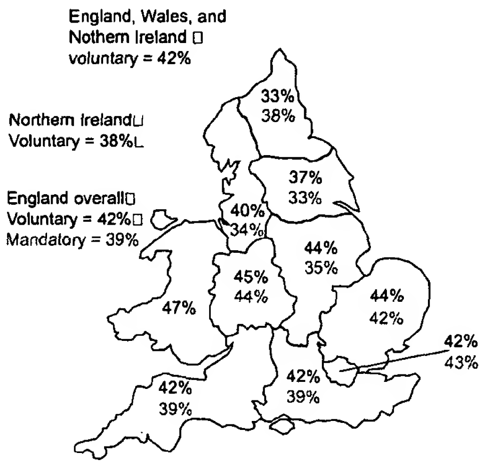
	Resistant	(%)	Sensitive	No information	(%)
Ciprofloxacin	423	53%	373	2431	75%
Erythromycin	906	39%	1395	926	29%
Fusidic acid	151	7%	1944	1132	35%
Gentamicin	71	4%	1684	1472	46%
Mupirocin	42	4%	1064	2121	66%
Rifampicin	23	2%	1325	1879	58%
Vancomycin	0	0%	1582	1645	51%

Figure 2 ***Staphylococcus aureus*** **bacteraemia voluntary reporting rates* per 100,000 population (95% confidence intervals), England, Wales, and Northern Ireland: July to September 2002**



*rates calculated using 2000 mid-year resident population estimates

Figure 3 Methicillin r sistance in *Staphylococcus aureus* bacteraemia reports*, England, Wales, and Northern Ireland: July t September 2002. MRSA as a p rc ntage f isolates whose susceptibilities were reported



Antimicrobial susceptibility

Comparing mandatory and voluntary reporting in England (table 1 and figure 3), complete information on methicillin susceptibility was received for the mandatory reporting scheme, whereas only 91% of voluntary reports had this information. There was little variation in the proportion of *S. aureus* bacteraemias resistant to methicillin between the two schemes in the West Midlands (1%), London (1%), and Eastern regions (2%), whereas in the East Midlands voluntary reporting indicated a higher rate of methicillin resistance than mandatory reporting - 9% higher.

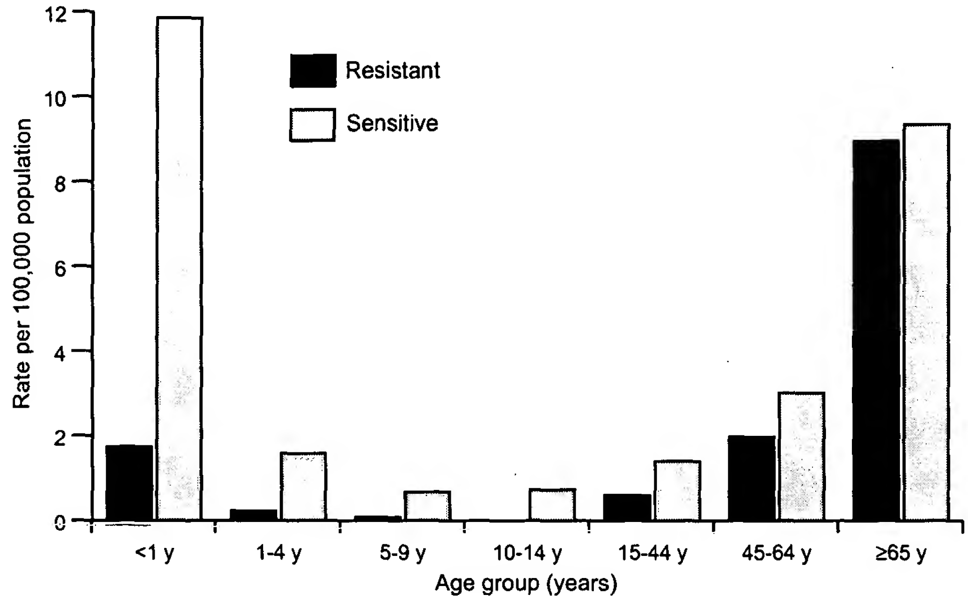
There was a significant lack of information on methicillin susceptibility under the voluntary reporting scheme in the North West (22%), North East (16%), and South East regions (12%). Only the West Midlands had complete information on methicillin susceptibility for the July to September 2002 quarter under the voluntary scheme.

Of the 2676 English voluntary laboratory reports of *S. aureus* bacteraemia with information on methicillin susceptibility, 1114 isolates (42%) were reported as resistant to methicillin. In Wales and Northern Ireland, methicillin resistance in *S. aureus* bacteraemias was reported in 47% and 38% of isolates respectively.

From the 4713 mandatory reports in England, 1827 (39%) isolates were reported as resistant to methicillin. The West Midlands had the highest percentage of methicillin resistant *S. aureus* (MRSA) isolates (45% voluntary and 44% mandatory) in England (table 1). The North East (33% voluntary and 38% mandatory) and Yorkshire and Humberside regions (37% voluntary and 33% mandatory) had the lowest proportion of methicillin resistant isolates.

Although the susceptibility of *S. aureus* to other antimicrobials (table 2) was also reported under the voluntary scheme, overall no more than half of the isolates contained such information. Of the reports that included susceptibility data, 53% of isolates were reported as resistant to ciprofloxacin, and 39% as resistant to erythromycin. Less than 10% resistance was reported to the remaining antibiotics listed in table 2. No reports of vancomycin resistance in *S. aureus* bacteraemia were received during this period.

Figure 4 Age-specific *Staphylococcus aureus* bacteraemia voluntary reporting *rates and methicillin susceptibility per 100,000 population, England, Wales, and N rthern Ireland: July to September 2002



Rates calculated using 2000 mid-year resident population estimates

Age distribution

The age-specific rate of MRSA (figure 4) was highest in those aged 65 years and over (9.0 per 100,000 population). This information is only obtainable from the voluntary reporting scheme and it is not included in the mandatory dataset. The proportion of methicillin sensitive *S. aureus* (MSSA) was higher than MRSA for all age-groups, with the highest rate reported for under one-year olds (11.9 per 100,000 population) followed by the 65 years and over (9.4 per 100,000 population).

Discussion

Three months of data (July to September 2002) from both the voluntary and mandatory reporting schemes for *S. aureus* bacteraemia are presented here, to allow comparison with earlier reports. Caution must be exercised when interpreting data from such a short time period. The data obtained under the two schemes (voluntary and mandatory) that have been analysed here as the voluntary scheme provides continuity with historic data and brings in added information, for instance on age and gender. It is also important to assess how the two systems compare, as the mandatory reporting system is likely to become disaggregate in the future (*ie*, based on routine reporting rather than quarterly aggregate reports). There were no substantial differences in the overall proportion of *S. aureus* bacteraemias due to MRSA in England under the two schemes: 42% methicillin resistance under the voluntary reporting scheme and 39% methicillin resistance under the mandatory reporting scheme.

Although methicillin susceptibility information was missing from 9% of reports from the voluntary scheme in England, this did not appear to strongly bias the results. For example, in London, there was a difference of

592 reports between the mandatory and voluntary schemes, although the proportion that were methicillin resistant was 43% and 42% respectively.

Compared to data from the previous report (1), where methicillin resistance of 41% and 44% from the mandatory and voluntary schemes respectively were observed, this quarter the percentage of MRSA was lower from both schemes (39% and 42%). Compared to voluntary data reported the quarter between January and June 2002 (1), there was a slight increase in the proportion that was methicillin resistant in the South West, West Midlands, and North West regions, whereas other regions showed a decrease in methicillin resistance. The results should be interpreted with care, as technical problems were encountered with reporting from the North West, and no information on susceptibility to methicillin for 61 isolates (22%) from the North West was available.

There were also problems with reporting from the Eastern region; it is likely that when the complete 2002 summary is published next year there may be some additional reports for this quarter.

Overall, susceptibility data for other antimicrobials was weak as there was a lack of information on more than 50% of the isolates. The incomplete information makes both comparisons and analyses with previous data difficult. The pattern of age-specific reporting rates of *S. aureus* bacteraemia is similar to the previous report (1), with the highest rates of MRSA in those aged 65 years and older.

A preliminary report on the surveillance of MRSA bacteraemia in Northern Ireland, from April 2001 to March 2002 has recently been published (2). The surveillance of *S. aureus* bacteraemias in Northern Ireland follows a similar strategy to that in England. The percentage of *S. aureus* blood cultures and patient episodes that were due to MRSA was 41.8% and 39.3% respectively for that period (2). Unpublished data received from Communicable Disease Surveillance Centre (CDSC) Northern Ireland (from this scheme) for the period July to September 2002, showed that 43% of *S. aureus* bacteraemias were resistant to methicillin. This is higher than the voluntary reporting rate for methicillin resistance of 38% shown in this report.

Acknowledgements

These reports would not be possible without the enduring weekly contributions from microbiology colleagues in laboratories across England, Wales, and Northern Ireland without which there would be no surveillance data. Feedback is welcome, and should be addressed to Georgia Duckworth email: (gduckworth@phls.org.uk). In addition, the support from colleagues within the PHLS, CPHL in particular, is valued in the preparation of the reports. These contributions are greatly appreciated.

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Immunisation

Last updated: 19 Dec mber 2002
Next update due: 23 January 2003

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Laboratory reports of hepatitis A in England and Wales: second quarter 2002*

During the second quarter of 2002, 316 laboratory reports of hepatitis A were made to PHLS. Sixty-one per cent (193) were men aged between 15 and 44 years table 1. Ten people acquired their infection abroad (country not stated) and 31 infections were in injecting drug users (IDUs). The incidence in these two groups has increased by about 60% since the previous quarter. The overall incidence of hepatitis A in the second quarter increased by 35% (111) compared to that of the first quarter. This was mainly attributable to the near 50% increase seen in men aged between 15 and 34 years.

The disparity between clinical notifications and laboratory reports improved in the second quarter of 2002 compared to 2001. In the first quarter of 2002, the number of laboratory reports actually surpassed the number of notifications. A total number of 316 cases of hepatitis A were laboratory confirmed, 4% more than formally notified. The greatest disparity was in the South West region with 83 laboratory reports made and 38 cases formally notified. This discrepancy is probably due to a case of under notification by GPs in the area. Disparity was also high in London, however, in this case the number of notifications greatly exceeded the number of laboratory reports. Twenty-seven cases were formally notified while only three laboratory reports were made. Under-reporting by London laboratories continues to impede surveillance, and potentially control measures, although populations at high risk are concentrated in the city.

Table 1 Laboratory reports of hepatitis A in England and Wales: second quarter 2002*

Age Group (years)	Apr-Jun 2002			
	Female	Male	Not known	Total
<1	–	2	–	2
1-4	3	3	–	6
5-9	8	7	1	16
10-14	4	3	1	8
15-24	23	82	–	105
25-34	25	86	1	112
35-44	8	25	–	33
45-54	13	5	–	18
55-64	5	1	–	6
≥65	4	5	–	9
NK	–	1	–	1
Total	93	220	3	316

* All data are provisional

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Laboratory reports of acute hepatitis B inf ction by ag group and sex England and Wales: s cond quarter 2002*

A total of 190 reports of acute hepatitis B infection were reported in the second quarter of 2002. The majority of cases (80%) occurred in the ±15 to 44 year olds table 1.

During the second quarter of 2002 injecting drug use was the main risk factor associated with hepatitis B infection, accounting for 20% (38/190) of individuals with known risk factors table 2. Hepatitis B infection associated with heterosexual exposure accounted for 12.6% (24/190), 4.7% in sex between men, and 7.9% in individuals with other risk exposures.

Table 1 Laboratory reports of acute hepatitis B infection by age group and sex England and Wales: second quarter 2002*

Age group (years)	Female	Male	NK	Total
< 15	–	–	–	–
15-24	27	24	–	51
25-34	19	40	–	59
35-44	14	28	1	43
45-54	1	15	–	16
55-64	–	11	1	12
> 65	–	5	–	5
NK	2	2	–	4
Total	**63**	**125**	**2**	**190**

* All data are provisional

Table 2 Laboratory reports of acute hepatitis B infection by exposure category in England and Wales Second quarter 2002*

Summary	Total
IVDU	38
Sex between men & women	24
Sex between men	9
Other identified risk	15
NRI	104
Total	190

* All data are provisional

Laboratory reports of hepatitis C infection in England and Wales: s cond quart r 2002*

A total of 1622 reports of hepatitis C infection were reported in the second quarter of 2002 table 1. Sixty-five percent (1053/1622) of the cases occurred in 25 to 44 year olds. Cases in males exceeded those in females.

Table 1 Laboratory reports of hepatitis C infection in England and Wales: second quarter 2002*

Group	Female	Male	NK	Total
<1	1	2	–	3
01-04	3	3	–	6
05-09	1	1	–	2
10-14	2	1	–	3
15-24	64	118	8	**190**
25-34	178	402	9	**589**
35-44	138	335	8	**481**
45-54	55	154	1	**210**
55-64	11	45	3	**59**
> 65	25	27	1	**53**
NK	8	15	3	**26**
Total	**486**	**1103**	**33**	**1622**

* All data are provisional

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Virus infections, England and Wales: laboratory reports, weeks 43-47/02

	Number of reports received						
Laboratory reports	**43/02**	**44/02**	**45/02**	**46/02**	**47/02**	**Total reports 43 -47/02**	**Cumulative total 2002**
Coxsackie A	–	2	1	1	2	**6**	**24**
Coxsackie B	1	1	–	2	4	9	**98**
Cytomegalovirus	8	27	16	13	22	**86**	**897**
Echovirus	–	–	–	1	14	15	**259**
Parvovirus B19	8	9	18	5	23	63	**1375**
Varicella zoster virus	1	4	6	4	11	26	**456**

Invasive mening coccal infections, England and Wales: laboratory r ports, we ks 33-36/02

	Method of diagnosis			Total reports 33-36/02	Cumulative total* 2002
	CSF and blood		Other sites		
	culture	non-culture**	culture		
Group A	–	–	–	–	1
Group B	27	18	3	48	1020
Group C	3	3	1	7	129
Group W135	4	–	–	4	68
Group X	–	–	–	–	3
Group Y	–	–	–	–	17
Group Z	–	–	–	–	0
Group 29E	–	–	–	–	0
Ungroupable	–	–	–	–	1
Ungrouped	–	2	–	2	95
Total	34	23	4	61	1334

* combined CDSC and Meningococcal Reference Unit data. ** latex antigen, microscopy, polymerase chain reaction.

Jpn. J. Infect. Dis., 54, 153-154, 2001

Laboratory and Epidemiology Communications

Prevalence of Norwalk Viruses in Southern and Northern Parts of Hiroshima Prefecture, Japan in 2000/2001 Season

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Communicated by Hiroo Inouye

(Accepted August 28, 2001)

Acute gastroenteritis in winter is caused mainly by viruses such as Norwalk virus, Sapporo virus, astrovirus, rotavirus, and adenovirus type 40/41 in Japan (1,2). Norwalk virus as well as rotavirus is the most important agent of viral gastroenteritis among pediatric patients (3), and a variation is seen from year to year in the prevalent types of Norwalk viruses (4,5). We report here on the prevalent types of Norwalk viruses in the southern and northern parts of Hiroshima Prefecture in the 2000/2001 season.

Seventy-six stool specimens from pediatric patients with gastroenteritis, excluding those with rotavirus, were collected from hospital sentinel stations, SE and SRC, located in the southern and northern parts of Hiroshima Prefecture between October 2000 and May 2001 (Fig. 1) and examined for gastroenteritis viruses. The detection of Norwalk virus genome was carried out by the method of RT-PCR with two primer sets of NV81/NV82/SM82 and Yuri22F/R (6). Viral RNA was extracted from 10% stool suspension in PBS(-) by using Trizol LS reagent (Life Technologies Inc., Grand Island, N.Y., USA). Twenty-one amplicons of Norwalk virus genomes were directly sequenced. Phylogenic analysis was performed using the Clustal W program (7) with 1000 bootstraps, developed by the National Institute of Genetics, Center for Information Biology and DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/), using 285 base nucleotide sequences from RNA polymerase region (nucleotide no. 4307 to 4591 as Camberwell strain). The phylogenic tree was drawn with Tree Explorer downloaded from its web site (http://evolgen.biol.metro-u.ac.jp/TE/TE_man.html). Sapporo virus and astrovirus genomes were detected using RT-PCR methods employing JV33/SR80 (8) and A230/AC1' (9) primers, respectively. Adenovirus type 40/41 was detected by ELISA kit (Meridian Diagnostics Inc., Cincinnati, Ohio, USA).

Norwalk virus and Sapporo virus were detected from 12 (30.0%) and 2, respectively, of 40 stool specimens collected in SE station. In SRC station, Norwalk virus, Sapporo virus, astrovirus, and adenovirus type 40/41 were detected from 14 (38.9%), 1, 2, and 3, respectively, of 36 stool specimens. Twenty of 21 Norwalk virus strains sequenced so far belonged to genogroup II and one belonged to genogroup I. As shown in Fig. 2, 7 of 9 Norwalk virus strains in SE station were classified as SE subset group which was related to Camberwell strain (GenBank accession no. AF145896). Eight of 12 strains from SRC station was classified as SRC subset which was related to OTH-25 strain (L23830). Similarities of the strains in SE subset with Camberwell strain were 92.9 to 93.6%. The strains in SRC subset showed 95.4 to 96.1% similarities with OTH-25 strain. The difference of nucleotide sequences in the strains of SE subset from Camberwell strain was recognized in the first nucleotide position of the codons, and the observed three amino acid differences were from Proline to Serine, from Isoleucine to Valine, and from Methionine to Valine. The strains of SRC subset were different in the third position of the codon from OTH-25 strain, but no difference was found in amino acid sequences. The prevalent types of Norwalk viruses differed regionally even within Hiroshima Prefecture in the 2000/2001 season.

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OECD WORKSHOP INTERLAKEN '98 ON

MOLECULAR TECHNOLOGIES FOR SAFE DRINKING WATER

ABSTRACTS

Waterborne disease education and control methods

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Increases in population over the past century have placed tremendous pressures on water resources of both the developed and developing world. These pressures include direct contamination from domestic, industrial, and agricultural wastes and less direct effects caused by climate change and other ecological disturbances. Population projections for the next century suggest that these pressures can only increase without appropriate intervention. Development, implementation, and maintenance of low cost, low technology water treatment systems are critical for reduction of global mortality associated with waterborne disease. Waterborne disease cannot be eradicated because of the variety of disease agents transmitted by water. Waterborne disease must be made reportable with active surveillance implemented as well as improved risk assessment methodology used. A newer method to become available is using molecular genetic based methods to detect the pathogens. Less scientific approaches are: policies related to waterborne disease are needed to integrate concerns and enable implementation of water treatment in both developed and developing countries. Governments, non-governmental organisations, institutions, and individuals with influence to affect public opinion must be educated about the social and economic burden of waterborne diseases. Training, education, technology transfer, and communication with the public via the media must be undertaken immediately. Society and the scientific community, working together as a team, to address the health risk issues should be the biggest priority to make international and national drinking waters safer.

The concepts of indicators and colony counts

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The aim is to produce and distribute safe water to consumers. Outbreaks of disease associated with mains drinking water are extremely rare, and there are very few incidents where there is a threat to public health. The volume of water treated is immense, distribution systems are extensive and there remains the potential for any failure in water quality to produce a health hazard for a large section of the

population. The measurement of the microbiological quality of water is based on the absence of the indicator organisms, principally coliforms and *Escherichia coli* in one hundred millilitre volumes of water in samples taken from water entering the distribution system, in service reservoirs, in the distribution system and at the customer's tap. The philosophy of this measurement approach is that it is very difficult to detect the gastrointestinal pathogens arising from sewage contamination, and it is therefore more logical to look for indicator organisms that arise from the human or animal gut that are non-pathogenic themselves, but indicate that sewage contamination has taken place to provide the indication that pathogens may be present. This philosophy can only be implemented by regular testing of many samples of drinking water. The colony count is used to determine deterioration in quality within the distribution system. It must be remembered, however, that the indicator organisms cannot predict when treatment failure has allowed protozoan parasites to pass through.

When problems do occur and the standards are not achieved, decisions have to be made. Accepting that the potential for harm could be considerable these decisions have to be made rapidly, accurately and confidently. The decisions relate to three areas:

- operational - correcting the problem that has led to the failure;
- public health - assessing the hazard, the potential risk and taking steps to minimise its effect;
- regulatory - reporting the incident to the regulating authorities and taking steps to minimise and eventually remove the effect.

There is now a requirement to evaluate the emerging new technologies and identify improvements that would further improve the protection of public health.

New approaches for the fast detection of indicators, in particular enzyme detection methods (EDM)

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The detection and enumeration of indicator organisms are of primary importance for monitoring the sanitary and microbiological quality of water. By incorporation of synthetic enzyme substrates into primary isolation media and kits, rapid enumeration and detection of indicator organisms can be performed (Enzyme Detection Method). The introduction of many of these media and identification tests has led to improved accuracy and faster detection of target organisms, often reducing the need for isolation of pure cultures and confirmatory tests.

The new enzymatic definition of total coliform bacteria is based on the presence of β -D-galactosidase, of *E. Coli* on the presence of β -D-glucuronidase and that of enterococci on the enzymatic action of β -D-glucosidase.

β -D-Glucuronidase is an enzyme that catalyses the hydrolysis of β -D-glucopyranosiduronic derivatives into their corresponding aglycons and D-glucuronic acid. The prevalence of this enzyme and its utility in the detection of *E. Coli* is reviewed. β -D-Galactosidase catalyses the breakdown of lactose into galactose and glucose and can be used for enumerating the coliform group within the family *Enterobacteriaceae*. Faecal streptococci and enterococci are useful as indicators of microbiological water quality, since they are common inhabitants of the intestinal tracts of humans and lower animals.

The use of B-D-galactosidase for enumeration of coliforms and B-D-glucosidase for identification of enterococci is described.

There is now a wide range of media available for detection and enumeration of indicator organisms in water and they are summarised in this paper. These enzymatic assays may constitute an alternative method for enumerating *E. Coli*, coliforms and enterococci, which is specific, sensitive and rapid.

Endemic versus epidemic waterborne disease

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The occurrence and severity of epidemic waterborne cryptosporidiosis may be inversely linked to the incidence of endemic waterborne *Cryptosporidium* infections. Because prior infections may reduce the severity of illness from subsequent *Cryptosporidium* infections, high levels of endemic waterborne infections may result in much of the population becoming resistant against severe cryptosporidiosis. Thus, the probability of detecting waterborne outbreaks in communities with high levels of resistance may be greatly reduced. A review of the epidemiological literature on waterborne cryptosporidiosis is consistent with this hypothesis. Also, in our previous studies, the prevalence of antibodies to two *Cryptosporidium* antigens was found to vary significantly between US communities. A second serological study of one community which is supplied by filtered surface-derived drinking water and another which is supplied by groundwater found large, statistically significant differences in both the seroprevalence to *Cryptosporidium* antigens and differences in the rates of seroconversion. Although additional serological studies of waterborne *Cryptosporidium* infections are needed, microbial risk assessment models and risk managers should begin to consider the role that protective immunity may play in reducing the frequency of large waterborne disease outbreaks.

Waterborne viruses and parasites: resistance to treatment and disinfection

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While most enteric bacteria are relatively easy to eliminate from contaminated waters by simple filtration and disinfection, viruses and parasites present a challenge that is often difficult to meet when source water is heavily contaminated. Rapid filtration alone cannot efficiently remove viruses and parasites; that can however be achieved by slow sand filtration and bank infiltration. To achieve removal by rapid sand filtration, an efficient coagulation process must be added in order to remove pathogens. Monitoring of removal has been performed by turbidity for decades, and waters with turbidities of less than 0.2 ntu can be achieved routinely. Particulate monitoring is currently being advocated as a better method for estimating the possible passage of the smaller protozoan cysts such as *Cryptosporidium*. Disinfection using ozone and/or chlorine can reduce to very low level most pathogens and indicators except for protozoans cysts, which are only effectively killed by extended chlorine contact or ozonation. Indicator micro-organisms such as coliforms (total and faecal), *Escherichia coli*, bacteriophages

(somatic and male-specific), spore-formers (aerobic and anaerobic) can be used at various stages of treatment or distribution to assess water quality. There are still many gaps in our knowledge of the behaviour of viral and protozoan pathogens and how indicators relate to viable pathogens. Current methods for pathogen detection rarely permit the detection of all micro-organisms, and while molecular methods appear promising, they are neither inexpensive nor a promise for better water quality and true protection of public health. All these aspects will be discussed in view of current epidemiological data on health effects of drinking water.

Molecular detection and identification of micro-organisms

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Detection and identification of micro-organisms are disciplines that complement each other in order to present a classification system that serves both applied and general microbiologists. Identification comprises the characterisation of a strain and the comparison of the data on that strain with data on previously classified and named strains. An organism can only be identified (i.e. shown to be identical with a known taxon) if that taxon is already known. Organisms which have not previously been isolated cannot be identified, must first be recognised as novel, and are then classified within the framework of the existing taxonomy. Identification is facilitated if the organisation stands phylogenetically isolated, i.e. if it contains genes and expresses gene products that are significantly different to those of its nearest phylogenetic neighbour. These unique properties may be targeted by non-culture dependent molecular identification approaches (probes, antisera, PCR products and so on). It may be concluded that the purpose and efficiency of a taxonomy is tested by its use in identifying organisms (Stackebrandt and Tindall, 1998). Should one encounter new species, then a key aspect of the existing classification is to what degree it allows one to predict the position of these of these organisms within the.classification system. The molecular affiliation of isolates to known species depends upon the availability of a clear cut discriminating tool which will be improved with the emergence of a wider range of fully analysed genomes. Nevertheless, the question whether a strain, analysed by nucleic acid pattern-generating approaches or even by genes or genome sequences, is part of a species or represents the nucleus of a new species will remain a core issue of prokaryote systematics.

Stackebrandt and Tindall (1998), Bacterial Systematics is the Cradle of Comparative Biology. Biology of the Prokaryotes, Thieme Verlag, Stuttgart (in press).

Immunological detection and quantification methods

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The quality of an immunological assay is largely dictated by properties of the antibodies incorporated. Antibody preparations vary greatly in the strength and specificity of their interaction with antigen (target

pathogen). There are three main kinds of antibody preparations: polyclonal antibodies, monoclonal antibodies (MAb) and recombinant antibodies. The different preparations will be discussed with particular reference to standardisation of tests and interpretation of results.

Enzyme immunoassays (EIA) are used widely to detect and quantify pathogens. They combine the specificity of antibody molecules with the amplification of antibody-antigen interactions by enzyme catalysis. There are many different EIA methods, and both qualitative and quantitative results can be obtained. EIA have been devised that are suitable for testing large and small numbers of samples. Fully automated tests can be done in the laboratory, or self-contained diagnostic kits can be used by non-specialists in less well equipped environments. Immunofluorescence and immunogold labelling methods rely on visualisation of the fluorochrome or gold-labelled antibody-antigen interactions by microscopy. These methods can provide useful information on the nature of the antigen in the test sample. The technical requirements of these methods will be described, and the results of tests to detect and quantify viruses and *Cryptosporidium parvum* oocysts will be presented.

Target genes for the identification and detection of potentially hazardous bacteria

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The discovery of a large number of bacterial toxins and other virulence factors such as adhesins and invasins has led during the last two decades to a significantly better understanding of bacterial mechanisms of pathogenicity and led to powerful molecular methods for the accurate detection and identification of pathogenic bacteria. Hence, the different medically relevant pathogenic types of certain bacterial species such as *Escherichia coli, Clostridium perfringens* and several *Pasteurella* sp. can be rapidly and accurately identified and clearly distinguished from their avirulent types by means of gene probes or PCR. However, for many pathogenic bacterial species no virulence factors or genes are known yet. For them, the differentiation of pathogenic types from less- or non-pathogenic types must rely on secondary markers or remains still impossible. We are currently developing gene probes for the detection of potential virulence genes in possibly most or all bacterial species. For this purpose, all known toxin genes were grouped to toxin gene families, according to their genetic relationships. From this knowledge, we have constructed broad range gene probes by cloning and amplifying the most characteristic and best conserved parts of the different toxin gene families. These sets of gene probes were first assessed for their capability to detect known toxin genes which were not included in the gene probes, under low to medium stringent hybridisation conditions. Subsequently, these probes were used to assess pathogenic bacterial species isolated from clinical samples as well as type- and reference-strains of several species from which no virulence genes were known yet. The method was validated further by isolation and characterisation of virulence genes from a few bacterial species that showed hybridisation signals to the probes. Using this approach, we have identified several new toxin genes from different pathogenic bacteria for which no toxins or other virulence factors were known yet. We prospect the broad range probes to be useful for the identification of waterborne pathogens.

Adaptation of methods to natural waters

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We probably know less than one per cent of the bacterial species which occur in the natural environment. In addition to this taxonomic ignorance, several unnamed species cannot grow on presently available culture media, and a number of bacterial cells of culturable species are in a viable and nonculturable state. Thus, detection of bacterial species by culture on agar media gives a grossly distorted view of the bacterial diversity in the environment (including water). Molecular methods targeting nucleic acids are the necessary tools for unveiling bacterial diversity. Ribosomal ribonucleic acids are universally present in bacteria, have diversely conserved portions of their sequences, and occur in about 30 000 copies per cell. Fluorescent oligonucleotide probes can be devised for *in situ* hybridization (FISH). Such probes can react with all bacteria, a given phylogenetic branch, a genus, or a single species. Different fluorescent labels can be used enabling multicolor reactions. Bottlenecks have been identified when FISH is applied to the detection of bacteria in water: (i) detection is strictly taxonomic (detection of *Escherichia coli* instead of coliforms), (ii) stressed bacteria are less reactive and often occur as tiny cells, (iii) some inorganic material may bind probes, (iv) the detection of a single indicator cell in 100 ml water is difficult under the microscope, (v) we want to be sure that detected bacteria are alive, and (vi) what about pathogenicity? Technological advances (gene amplification, signal amplification, viability tests, cell concentration, image analysis) attempt to answer these points.

"Old" versus "new" methods

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The microbiological monitoring of water involves methods for the detection of bacteria, viruses and protozoa. Many of the methods used are non-specific, are inaccurate and time consuming. Whilst technological advances have been made which have facilitated important changes to the way in which microbes may be detected, there is a particular difficulty in validating many of these procedures. Furthermore, there is little consensus in the way in which methods should be validated when researchers, routine laboratories and regulators discuss this issue. Often the difficulty in validating methods is due to the fact that more modern molecular methods may identify either a broader or narrower group of organisms than conventional tests.

In order to improve our ability to monitor the microbiological quality of water, new approaches must be embraced, but before this can occur, there must be some guidance given internationally on validation procedures and required performance characteristics. Specific examples of problems with the implementation of new microbiological methods will be discussed with reference to both regulated and non-regulated testing of bacteria and protozoan parasites.

Analytic methods in support of the safe drinking water and clean water in the United States

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The US Environmental Protection Agency (EPA) is charged with establishing and administering regulations to support major environmental laws including the Clean Water Act (CWA) and the Safe Drinking Water Act (SDWA). SDWA specifies that EPA promulgate regulations for drinking water contaminants which may cause adverse human health effects and which are known or anticipated to occur in public water systems. EPA determines risk-based non-enforceable Maximum Contaminant Level Goals (MCLG) for both chemicals and microbes. Enforceable Maximum Contaminant Levels are set as close as practicable to the MCLG after consideration of costs, availability of treatment options and of suitable analytic methods. The CWA mandates that EPA develop risk-based criteria and analytic techniques that enable States to enforce protection of water quality. Water bodies are designated for specific uses; such as, drinking water source, fishing, recreation, etc. Standards to protect these uses are promulgated by States based on assessment of human or aquatic life risk, and analytic techniques are used to monitor compliance. EPA is currently focusing major resources on updating and standardisation of techniques for quantification and speciation of microbial contaminants of fresh, estuarine and marine waters. The process of acceptance of methods will be described.

What does it take to bring a method to use?

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The United Kingdom recommends the use of methods described in "The Microbiology of Water 1994 – Part 1 Drinking Water" referred to as Report 71. Laboratories may use alternative methods providing they have been shown capable of equivalent or better performance by means of inter-laboratory trials. Key issues are the need for unequivocal scientifically valid definitions of the organisms being detected, and acceptable criteria for determining equivalence of methods. The Drinking Water Inspectorate does not regard itself as a source of expertise but relies upon expert advice to support its decision making. There is a serious lack of such advice when it comes to new methodologies.

R&D priorities and technical issues for molecular technologies for safe drinking water

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The research and development priorities for applying molecular technologies for safe drinking water begins with the identification of what needs to be monitored based upon risk analyses. Specific pathogens of concern will vary among water sources, making it difficult to harmonise the risk assessment process. Once specific targets are identified, the technical issues for molecular methodologies will be sensitivity, specificity, and cost. The detection costs must be low and the benefits

must outweigh the continued use of conventional methods; the molecular methods must be specific for the micro-organisms of concern, which means specifically being able to detect live organisms capable of causing disease; and the sensitivity must be adequate to provide protection against waterborne disease, which means being able to concentrate large volumes of water and overcome interfering factors so as to detect very low numbers of micro-organisms.

Policy and administrative issues

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Despite substantial advances in recent years and especially during the IDWSSD, 1981-1990, ensuring access to safe drinking water remains a major challenge to public health. Around 20 percent of the world's population lack access to safe drinking water, leading to both a burden of disease and constraints to socio-economic development. Well-documented improvements in water supply provision began in some countries with the sanitary revolution of the middle of the last century and continue. Recent years have seen re-awakening of recognition of the importance of the microbiological quality of drinking water world-wide. This has been driven by diverse factors, including recognition of new pathogens, more sensitive approaches to outbreak detection and universal problems with ensuring safety of small supply systems. Changes in legislative approaches towards ensuring microbiological quality are ongoing and include a process of revision of the microbiological component of the WHO *Guidelines for Drinking-water Quality,* which has recently been initiated. New, especially molecular methods for safe drinking water may assist in these changes and thereby contribute to increased sectoral efficiency and the increased provision of safe drinking water. However these methods highlight questions regarding the public health significance of very low dose encounters and encounters with "survival forms" of bacteria. They also provide new challenges, especially resulting from internationalisation and inter-laboratory comparability.

Sensitive detection techniques – financial implications for water customers

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With the development of more sensitive detection techniques, more consideration may need to be given to the interpretation of any findings and the application of results from these tests. It may not be appropriate to set quality standards for either drinking water or the environment on the basis of the most sensitive techniques available.

In England and Wales, there has been a similar debate relating to improved analytical techniques for chemical compounds, such as the detection of pesticides and pesticides residues in drinking water. The adoption of the precautionary principle has led to stringent standards being set for the maximum permitted level of pesticides, in effect a surrogate zero level. The current EC standard for drinking water is 0.1 microgrammes per litre which disregards the relative toxicities of this heterogeneous group of

compounds. However in England and Wales the adoption of this standard has led to capital investment of over £1000m. It is highly debatable whether this level of expenditure can be justified on toxicological grounds.

Before standards relating to more sensitive detection techniques are introduced, there is a need to assess the impact on water customers, balancing any risks to public health with the potential costs or impact on the cost of providing drinking water. These tests may also influence the setting of standards relating to environmental water quality. In many cases specific rapid tests, particularly for the detection of potentially pathogenic micro-organisms, could well be advantageous to customers. However, there is a need to review the costs and benefits alongside similar risks prevalent elsewhere.

Detection and identification of indicator bacteria using rRNA targeted probes

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Molecular identification of micro-organisms improved enormously during the last decade. Especially, primary structures of ribosomal RNAs (rRNAs) have been found to harbour information about phylogeny and identity of bacteria. PCR-amplification of genes for 16S and 23S rRNAs, their sequencing, and computer aided analysis of large sequence databases, enable the finding of short signature sequences unique in groups of bacteria or even single species. Therefore oligonucleotides, complementary to those unique signatures, are ideal tools for rapid detection and identification of concerning organisms. In this study, a set of 16S and 23S rRNA targeted probes has been designed to identify groups of enterococci and intestinal streptococci, and to differentiate between important species.

These probes have been applied in different hybridization techniques which might be suitable for routine microbiological quality control of drinking water. Both a reverse hybridization assay in microtiter formate for the detection of PCR-amplified rRNA-genes, and the "Fluorescence Microcolony Hybridization", which is a rapid and specific visual method for the detection and identification of indicator bacteria, will be introduced.

Detection of viable pathogenic bacteria from water samples by PCR

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The Polymerase Chain Reaction (PCR) is a molecular technique which can be used to identify specific bacterial strains within a mixed population. A detection method has been developed for strict and opportunistic pathogenic bacteria (*Salmonella*, enterohemorragic *Escherichia coli* and *Aeromonas hydrophila*) in raw and treated water. This method is composed of a bacterial DNA purification step followed by PCR detection. Compared to the traditional culture techniques, this method has an enhanced specificity and sensitivity. Furthermore, the simple and rapid protocol of the proposed technique

provides results at a fraction of the time required by the traditional culture techniques (24 hours compared with two to six days).

However, unlike the culture methods, detection by PCR does not provide information related to the viability of the bacteria, since the detected bacteria can be viable and cultivable, viable but non-cultivable, or dead. The viability concept is very important for interpreting the detection of pathogenic bacteria in relation to public health issues. To overcome this limitation, an indirect approach has been developed for assessing the viability of PCR-detected bacteria from water samples. This method is based on the analysis of each sample before and after a 20-hour culture step in a non-selective medium: an increase in the PCR response after cultivation indicates the occurrence of bacterial multiplication and thus demonstrates the viability of the detected bacteria.

This new protocol allows the simultaneous detection of several viable cultivable pathogenic bacteria by PCR from water samples.

Bacteroides spp. as alternative indicator organisms: monitoring through PCR 16S-rRNA amplification

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Some bacterial species of the *Bacteroides/Prevotella* group have been proposed as "indicator organisms" for the evaluation of faecal pollution, alternatively to coliforms. Comparatively, the genus displays several advantages; while outnumbering coliforms by about 100 times, different *Bacteroides* species colonise human and animal digestive tracts, therefore behaving as "indicators" of the type, as well as of the origin of the contamination, in addition to its intensity.

A tentative "state of the art" on the use of *Bacteroides* species as "indicator organisms" is presented. Results from ongoing R&D on the use of PCR for detection and counting of *Bacteroides/Prevotella* cells in faeces, waters, wastewaters and treated effluents are shown. Primer selection, polymorphisms, cross reactions and sensitivity for live and dead cells, as well as prospects and limitations, are discussed.

PCR detection of *E. coli* after disinfection with UV or ozone

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Monitoring of enteric pathogens is currently conducted by filtration and cultivation of indicator bacteria on selective media. But these traditional procedures are time consuming and not always sensitive enough to exclude any risk of contamination by quiescent bacteria. Alternative microbiological methods should be developed and tested to complement or even replace the cultivation techniques.

The PCR offers the potential for specific, sensitive, and fast detection of pathogens. In our study, a PCR

procedure with two specific 16S rDNA primers for the detection of *E. coli* was developed and compared to enumeration by culturing. Testing of dilution series of bacterial suspensions showed an about ten times greater gene copy number in PCR than CFU, in agreement with the bacteria counted under the microscope.

The response of the PCR was tested with suspensions exposed to ozone or UV light, and the results compared to enumeration by cultivation. Especially the UV disinfected suspensions produced a variety of not defined amplification products that make the interpretation of the results difficult. These side products, appearing as a smear on the gels, are an effect of the high *E. coli* background in the samples. Still, the PCR method showed greater sensitivities up to several orders of magnitude than cultivation. These results suggest that the PCR detects also dead cells with at least partially intact DNA and is therefore not suited for measuring the success of such water treatments.

Application of new methods in regular water hygiene control

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In the microbiological laboratory of the waterworks of the city of Mainz, experience has been gained concerning use of recently developed immunological and molecular biological methods in water hygiene control. As alternatives for the standard detection of *E. coli* / Coliforms, besides commercial rapid tests (e.g. Colilert, Idexx; or Chromocult media, Merck), a self-developed rapid immunological test (ELISA-Systems Enterobacteriaceae, Riedel-de Haën) was utilised, that recognises Enterobacteriaceae including indicators and pathogens. It has been approved as German Pre-Standard. Another self-developed immunological test is suited for the detection of pre-cultured *Legionella*. The colony-blot assay (ELISA-Systems Legionella, Riedel-de Haën) is a feasible alternative to the usual time-consuming identification steps. In Germany there has been no standard method for *Legionella* so far, but the colony-blot assay is mentioned in the actual ISO draft for detection of *Legionella*. A gene-probe test-kit (Applied Biosystems) has also been employed with good results. A molecular biological test for hygienically relevant bacteria based on 23S rDNA-targeted oligonucleotide probes is still under development. It is aimed at the simultaneous detection of different species from a single enrichment step. At present, the method has been tested for enterococci and *E. coli*. To estimate the risk of bacterial regrowth in distribution systems, investigations on biofilm formation are conducted by use of *in situ* hybridization techniques.

An intensive information and co-operation policy towards the local health authorities promoted the acceptance of results obtained with new methods, provided that standard procedures are applied in parallel. But in spite of extensive interlaboratory evaluation studies and obvious advantages, the rigid views of some hygiene experts have hindered the consideration of new methods in the Federal German Drinking Water Commission yet. Therefore concerted action on an international level could probably support the approval.

From design through to routine implementation of a PCR system for *Listeria monocytogenes*

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A PCR method for the detection of *Listeria monocytogenes* was designed. A region of DNA from the 23S rRNA gene was chosen to identify the *Listeria* genus and the listeriolysin O gene to identify the monocytogenes species. The amplification procedure was optimised and tested against all *Listeria spp*, a range of related genera and unrelated genera (46 samples). As viability was essential and speed important, samples were grown in two broth steps, the cells washed and the DNA extracted by heat blasting followed by amplification. This test was trialed on a large (110 samples) validation study using a variety of foods. The PCR system obtained 300 per cent more positives than the conventional method. Finally 70 ham samples were tested for *Listeria monocytogenes* comparing this PCR system and an immunoassay system. All PCR and immunoassay positive results were confirmed by direct culture. No false negatives were obtained by the PCR, however the immunoassay system gave 6 out of 70 false negative results. The PCR system has been passed onto our food testing laboratory to be trialed as an initial screening tool for all food samples for *Listeria monocytogenes*, since it is accurate, quicker, more sensitive, and cheaper than the traditional culturing method. However, until the test has official recognition, all positives will then be confirmed by culturing.

The Chem*Scan*® RDI, a real time and ultrasensitive microbial analysis system

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Within the pharmaceutical, cosmetics and drinking waters industries, in process control for the quantification and trending of microbial contamination is a vital part of manufacturing.

The ability to detect potential problems as they arise is the key to improve quality and reduce wastage.

The standard agar growth plate still holds for the detection and enumeration of viable micro-organisms, even though results are dependent upon the incubation period, typically 3-5 days, and up to 14 days for stressed organisms.

Rapid technologies for detecting microbial contamination are increasing, but most of them have sacrificed sensitivity for speed and are not suitable in many of the most critical applications, where the ability to detect very low numbers of microbial cells is of key importance.

Chemunex has recently introduced Chem*Scan*® RDI, a microbial analysis system which has the capability to provide ultrasensitive results down to one cell within 90 minutes of sampling without requirement of any microbial culture.

The system combines the fluorescent labelling of single viable micro-organisms and a solid phase laser scanning analyser. The system directly detects bacteria, fungi including spore-forming species, yeasts, anaerobes and extremophile bacteria. An automated epifluorescence microscope connected to the instrument allows an immediate visual confirmation.

Multi-site validation has shown Chem*Scan*® RDI to be at least as sensitive as the plate counts method.

New real time applications based on the use of specific substrates for known intracellular enzymes, fluorophore linked monoclonal antibodies and nucleic acid probes provide new opportunities for the specific detection of pathogen and questionable micro-organisms.

Molecular techniques for the detection of enteroviruses in water

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Enteroviruses consisting of the group of Polioviruses, Coxsackieviruses group A and B, Echoviruses and some numbered Enteroviruses may pass drinking water treatment steps without being inactivated and are therefore a public health concern. The traditional detection of Enteroviruses requires the laborious and time-consuming propagation of the concentrated viruses in specific cell culture. Faster and more sensitive molecular technology is now available. The Polymerase Chain Reaction (PCR) can be used for the detection of very low viral titers and can be accomplished within a few hours. Part of the enteroviral genome is the 5-noncoding region (5'-NCR) which contains sequence homology boxes highly conserved among different enteroviruses. A group-specific detection becomes possible by using oligonucleotides out of the 5'-NCR. A big disadvantage of these methods is that it is not possible to discriminate between infectious and inactivated viruses. However, a selective detection of infectious viruses is necessary for the correct assessment of drinking water quality. Currently we are working on the development of methods for the detection of replicative forms of the viral RNA indicating viral replication on cell culture.

Molecular characterisation of enteric viruses detected in surface water

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To recover waterborne enteric viruses, the permanent cell lines RD6, MA104, and MDBK were each inoculated with one-third of the concentrate of 1L samples obtained from local rivers. Out of 98 samples, 24 were found to contain cytopathogenic viruses. These were identified as human or bovine enteroviruses (6 and 1 isolates), reoviruses (12 isolates), rotaviruses (3 isolates), and mixtures of reo- and rotaviruses (2 isolates). The rotaviruses were invariably isolated in MA104 cells. When evaluated with RT-PCR and using genotype-specific primers, 4 of the 5 rotaviruses were recognised as members of the rare G8-serotype. Sequencing of the relevant gene from one of these isolates confirmed the similarity of this virus with known rotavirus G8-types. The four rotavirus G8-types were recovered in the year 1996 during a 6-week period from two locations on the same river. This is the first report on the prevalence of this virus type in Switzerland.

The global prevalence of G8-type rotaviruses is generally thought to be low. Few such viruses have been isolated from cattle and even less from humans. Calf diarrhoea is most regularly associated with G6-, less often with G10-, and least with G8-genotypes. Irrespective of the host(s) involved, the data indicate that the river under study was at least temporarily highly contaminated with G8-type rotaviruses. Additional molecular epidemiological studies will help to identify the original host(s) of these environmental contaminants.

Molecular methods for the detection of small round structured viruses in water

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Small round structured viruses (SRSV) are positive stranded RNA viruses. In the Netherlands, SRSV are associated with a significant part of the outbreaks of intestinal illness. SRSV have been implicated as the causative agent in numerous food- and waterborne outbreaks of intestinal illness world-wide. The aim of this study was to develop and evaluate a method for the detection of SRSV in sewage water samples by RT-PCR, to be able to determine the significance of the risk of waterborne transmission.

Sewage samples were taken at the site and during the course of two confirmed outbreaks of SRSV associated gastro-enteritis. Sewage was first concentrated by a filter adsorption-elution method. The concentrate was purified with a PEG/dextran sequestration, and RNA was extracted with silica beads. SRSV-specific primers were used which anneal to a part of the SRSV genome within the viral RNA polymerase gene (ORF1) and were shown to detect 85 per cent of a panel of different SRSV strains.

In one outbreak, the sewage sample taken at the time of the outbreak was positive in the RT-PCR. The positive PCR products were sequenced, and the sequence of SRSV from the sewage sample correspond with the sequence of the stool samples of the patients.

At the second outbreak, SRSV was detected in the sample that was taken one day after the outbreak was reported, but not in subsequent samples.

This study shows that this concentration and purification method is applicable for virus detection by RT-PCR on sewage samples. Even though RT-PCR can not discriminate between infectious and non-infectious viruses, it offers a rapid way for screening different kinds of samples.

This method will now be used to determine the occurrence of SRSV in the aquatic environment.

Detection of *Cryptosporidium* oocysts in water: Comparison of the conventional microscopic immunofluorescence method with PCR and TaqMan PCR

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Cryptosporidium parvum is a protozoan parasite which is transmitted via the faecal oral route and can cause diarrhoea in man and animals. The ca. 5μ m sized oocysts which are shed with the faeces of infected individuals can contaminate surface waters and inadequately protected ground waters and are extremely chlorine resistant. The conventional microscopic method for the detection of *Cryptosporidium* oocysts in water samples has been compared with molecular detection methods consisting of 1) a DNase treatment of immunomagnetic separation (IMS) to avoid false positive results from free DNA, which may be present in a sample material; 2) an *in vitro* excystation protocol to assess the viability of the organisms; and 3) the polymerase chain reaction (PCR or TaqMan PCR) for the detection of gene sequences of excysted soprozoites. The sensitivity of the molecular detection methods was comparable to the sensitivity of the microscopic method when spiked river of drinking water samples were examined. It was, however, inconsistent over time and sometimes showed inexplicable outfalls. TaqMan PCR with product detection in a Perkin-Elmer-LS 50B luminescence photometer was at least as sensitive as PCR with product detection in ethidium bromide stained agarose gels. Quality control for the microscopic method was done by participation in the External Quality Assessment Scheme for *Cryptosporidium* detection in water samples, performed by the PHLS, UK. The PCR methods would be most valuable to control presence-absence standards.

Clustering of *Acanthamoeba* isolates by means of mtDNA digestion patterns

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Small free-living amoebae of the genus *Acanthamoeba* are known both to cause conjunctivitis occurring predominantly among contact lens wearers and to act as a vehicle for *Legionella* in aquatic environments. Whether or not all strains of this genus have a pathogenic potential, and whether or not distinct pathogenic strains occur only in specific habitats, are questions that need to be resolved. Knowledge of the genetic identity of pathogenic strains will give new insights into these questions. We used mtDNA restriction fragment length polymorphism (mtDNA RFLP) analysis to study the molecular epidemiology of *Acanthamoeba* isolates. Ninety isolates from human eye infections from 15 countries were clustered into distinct genotypes. Closely related digestion phenotypes (sequence difference = 0.1-1.5 per cent) were integrated into a single genotype while phenotypes with greater than 4.76 per cent difference were considered distinct. Approximately 80 per cent of the human isolates studied fell into seven of 22 genotypes, indicating that virulence may be associated with specific clusters of cladistic groups of *Acanthamoeba*. This technique is useful for large-scale surveying of this particular pathogen.

Species differentiation of *Giardia* by PCR

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Using random amplification of polymorphic DNA (RAPD), a band present with *G. muris* but not *G. intestinalis* DNA was detected. This band was cloned and sequenced and this facilitated the selection of a pair of primers specific for *G. muris*. Since *G. muris* does not infect humans, the use of these primers in conjunction with primers specific for *G. intestinalis* should enable water to be determined as safe or unsafe for human consumption with respect to the species of *Giardia*.

Toxic cyanobacteria in resource waters: monitoring of their occurrence and of the toxin detection

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A two-year monitoring of three locations in rivers of the Paris area has demonstrated the permanent occurrence of Microcystis, Oscillatoria, Synechoccus, Synechocystis and Anabaena, *M. aeruginosa* and *O. agardhii* being the most frequently present.

In order to progress in the evaluation of the toxic risk associated with these micro-organisms, a two-step approach is being developed.

- The first step comprises the observation of microalgal samples collected every month in parallel with the detection of microcystins, the most abundant toxins, with a serine-threonine protein phosphatase assay; capillary electrophoresis is used to confirm the presence of these hepatotoxins.
- The second step has started with the isolation of algal strains in axenic cultures for the development of molecular probes.

Mathematical models for the distribution of organisms in water distribution systems

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An important issue to consider in the interpretation of microbiological sampling data from drinking water supplies is the large variation in counts. The variation is both temporal (i.e. from day to day) and spatial (i.e. differences between zones and within a given zone). The sources of variation include regrowth in distribution, breakthrough from treatment works, ingress of contaminated water and differences in age/history of the water within a zone. Some of the variation in plate count and coliform occurrences can be modelled on the basis of changes in chlorine concentration and temperature. For coliforms, and indeed some pathogens, it is not unusual to record a series of zero readings and a small proportion of positive samples, some with high counts. Those count data, including the zeros, can be modelled using the negative binomial and Poisson-lognormal distributions which allow for greater variation than the Poisson distribution. The large degree of variation in micro-organism densities raises

the question of what a series of zero readings means. First, a series of zero readings does not exclude the existence of regions within the supply with high counts. Second, some zeros may have been "near-misses". Indeed, there have been waterborne outbreaks where pathogens were never detected in the water supply. Large volume sampling could be used to distinguish "near-miss" zero samples from "secure" zero readings. This paper explores how spatial/temporal variation of micro-organism densities influences compliance with standards and the risk to public health from waterborne pathogens.

Detection of microbes in water treatment stations in Japan

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The drinking water in Japan should satisfy the criteria indicated in the Water Quality Law of the Ministry of Health number 69 issued on December 21, 1992. According to the law, the bacterial colony count per 1 ml on the normal agar plate should be less than 100, and that of *Escherichia coli* species per 1 ml should be below the detection level. The detection should follow the procedures appearing in Annex 1. The water also should satisfy the restrictions regarding 27 toxic chemicals.

The data on drinking water presented in this document is based on the survey compiled by Dr. S. Yamai, Hygienic Laboratory of Kanagawa Prefecture. The survey was performed twice, in July and October 1997. The data on the river water (November 1996-February 1998) were derived from the reports published in the Infectious Agents Surveillance Report (ISSN 0915-5813). The survey was conducted by Hygienic Laboratories of Kanagawa Prefecture, Yokohama City, Kawasaki City, Shizuoka City, Osaka City and Tottori Prefecture.

The case of protozoa: measures for water safety and reuse, Mexico

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Guidelines on "zero risk" (faecal coliforms/100 mL) have been conceived as a "nil threshold", to protect public health against "waterborne" diseases. Treated water complying with bacterial quality standard is being used for multiple purposes, and as a way to preserve "fresh" supplies (SAT) for increasingly thirsty populations. A growing number of reported outbreaks world-wide (i.e. enteric diseases) associated with "pristine" waters, are emerging as warning signals against overly simplistic thinking.

We present preliminary data on the health impact from a water and land reclamation project in a large suburb of Mexico city (Xochimilco). The area receives the effluent of a secondary treatment plant (PTCE), which is to be upgraded and used for crop irrigation (i.e. flowers, maize, vegetables) and aquifer recharge. Two cross-sectional surveys were carried out during the rainy and dry seasons, 1996-1997. The health outcomes included diarrhoeal diseases, as well as protozoal infections (i.e.

Cryptosporidium parvum and *Giardia lamblia*). Data from the population were collected by means of structured interviews and parasitological tests. Water samples were obtained from selected points (effluent of PTCE and canals). Water quality indicators were FC/100 mL and cysts of *G. lamblia* and oocysts of *Cryptosporidium*.

A total of 350 children under five years of age were involved in the study. Analysis is in progress, but available results from the rainy season study showed a prevalence of diarrhoeal diseases of 14.5 per cent, whereas infection with *G. lamblia* and *Cryptosporidium* affected 9 per cent and 5.4 per cent, respectively.

Data from water samples indicated that the effluent from the treatment plant complies with current water quality regulations (0 FC/100 mL). Nevertheless, high concentrations of FC/100 mL throughout the network of canals suggested that local sewage discharges (i.e. illegal settlements) are "diluting" the expected effect of the treatment facility. In addition, cysts of *G. lamblia* have been detected from the effluent and from a large wells. Research needs and policy making will be discussed.

Prevalence of *Cryptosporidium* and *Giardia* in waste- and surface water in Israel

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Cryptosporidium and *Giardia* have been found responsible for the occurrence of waterborne outbreaks in various parts of the world. Both parasites can originate from human and animal wastes. *Giardia* and *Cryptosporidium* share common characteristics, such as persistence for a long time under adverse environmental conditions and great resistance to disinfection processes, especially chlorination. These properties enhance their potential for transmission through water and food. The objectives of this study were to determine the prevalence and concentration of *Giardia* cysts and *Cryptosporidium* oocysts in raw wastewater in Israel. Furthermore, to evaluate the removal efficiency of the parasites by secondary wastewater treatment. The prevalence and concentration of *Cryptosporidium* and *Giardia* in surface water sources in northern Israel were evaluated. All tested raw wastewater samples were found positive for both parasites at a concentration of 10^3-10^4 *Giardia* cysts/litre and 10^2-10^3 *Cryptosporidium* oocysts/litre. This survey represents 10 cities in Israel, including the central wastewater treatment plant of the Dan Region. The parasites' concentration was not influenced by seasonal differences. Secondary wastewater treatment (activated sludge) resulted in the removal of 99 per cent of the *Giardia* cysts and 90 per cent of the *Cryptosporidium* oocysts. A preliminary survey conducted in the Jordan river and at the entrance of a small stream in lake Kinneret revealed that both locations are contaminated with protozoan parasites at levels between 20-130 oocysts/100 litres for *Cryptosporidium* and 20-210 cysts /100 litres for *Giardia*. It is important to bear in mind that the recovery efficiency of the concentration and purification technique is low; therefore, the real number could be higher. The results of this study indicate that wastewater contains high concentrations of protozoan parasites which can be introduced to drinking water sources.